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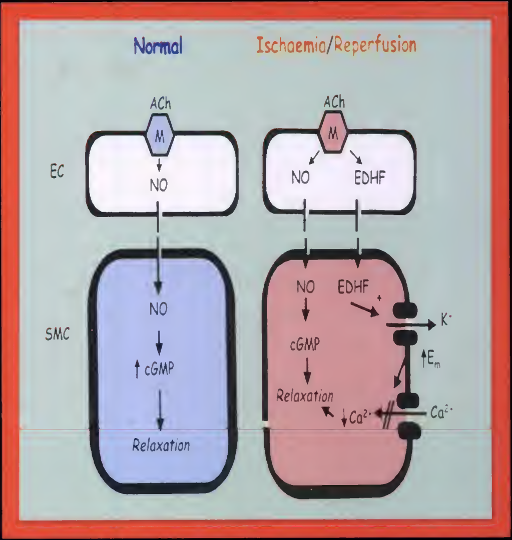
CURRENT PHARMACEUTICAL DESIGN



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CURRENT PHARMACEUTICAL DESIGN

Volume 5, Number 12, December, 1999

Cardiovascular and Hematological Agents

Guest Editors: James M. Hamby/Annette M. Doherty

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Current Pharmaceutical Design, 1999, 5, 965-986

Development of Heparin Antagonists with Focused Biological Activity

Shilpa Shenoy[1,2], Michael Sobel[3] and Robert B. Harris[1,*]

[1]Commonwealth Biotechnologies, Inc., 601 Biotech Dr., Richmond, VA, 23235, USA

[2]Current address: National Cancer Institute, Frederick, MD, USA

[3]Syracuse VA Medical Center, Surgery Service 112, 800 Irving Ave, Syracuse, NY 13210-2716, USA

Abstract: Heparin, a complex glycosaminoglycan, has long been used to temporarily render the blood incoagulable during extracorporeal circulation, cardiovascular surgery, and other arterial interventions. But bleeding complications are especially common when the arterial tree is violated, occurring in as many as 10-15% of cases. For cardiovascular surgery and many related interventions, protamine has long been the standard antagonist when acute and complete neutralization of heparin's anticoagulant effect is necessary. Protamine's efficacy is related in part to its total net cationic charge, but unfortunately so is its toxicity. For these reasons, there is renewed interest in developing heparin antagonists which will replace the use of protamine. At Commonwealth Biotechnologies, Inc., we have used a rationale design approach for the preparation of a family of low molecular weight helix peptides which bind heparin with high affinity. For each of the new compounds, we have assessed their ability to bind heparin using isothermal titration calorimetry and circular dichroism spectrometry and have examined potential complexes formed with the anticoagulant pentasaccharide unit of heparin using molecular modeling techniques. The biological potencies of these compounds were assessed in *ex vivo* experiments where their ability to compete with antithrombin for binding heparin was determined. The best of the compounds, designated HepArrest^{TM}, is highly effective in reversing heparin-mediated and HepArrest is a safer drug than protamine because of reduced adverse hemodynamic side effects compared with those associated with protamine. HepArrest binds low molecular weight heparins and causes reversal of anticoagulation by low molecular weight heparins, as determined by activated partial thromboplastin time, thrombin time, or factor Xa neutralization assays. These highly promising preclinical results indicate that HepArrest is a novel heparin neutralizing agent that may well fill a substantial unmet need for vascular surgeons and cardiac anesthesiologists who perform coronary artery bypass grafts and several other major vascular surgeries, as well as for cardiologists and interventional radiologists.

BACKGROUND

The major anticoagulant agent used in virtually all surgical procedures is heparin. Although heparin is best known for its anticoagulant and antithrombotic properties, recent work has shown its therapeutic potential in the control of the inflammatory process [1].

Heparin belongs to the family of glycosaminoglycans, which are a family of structurally distinct polyanionic, complex carbohydrates which include heparin, heparan sulfate, chondroitin sulfate, dermatan sulfate and hyaluronic acid [2]. These polysaccharide chains are composed almost entirely of repeating disaccharide units consisting of an amino sugar, either D-glucoseamine or D-galactoseamine, and uronic acid. The biosynthesis of these molecules does not follow a blueprint, and a heterogeneously sized population of carbohydrate chains with variable sulfation patterns and conformations are found *in vivo* [3]. The most complex class with regard to its microheterogeneity in composition and structure is heparin, and it is this class which appears to exhibit the greatest biological diversity.

In most mammals, heparin is localized to the mast cells, but in some species, heparin is found in mucosal and other membranes [4]. Regardless, heparin ranges in molecular weight from 5,000 to

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[*] Address correspondence to this author at the Commonwealth Biotechnologies, Inc., 601 Biotech Dr., Richmond, VA, 23235, USA; Email: rharris@cbi-biotech.com; phone 804-915-3480; fax 804-915-3830.

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50,000, with an average molecular weight of 15,000 [the so-called "heparin monomer") [5].

The anticoagulant activity of heparin is related to its interaction with two proteins of the serine proteinase inhibitor (serpin) class, antithrombin and heparin cofactor II [6]. Binding of heparin to antithrombin (AT)[1] greatly accelerates the binding of AT to thrombin, causing inhibition of thrombin-catalyzed formation of fibrin from fibrinogen. The initial event in AT/heparin binding is recognition of a specific pentasaccharide "unit structure" within the heparin polymer (found only in about 30% of total pool of polysaccharides) [7] (Fig **1**). However, to achieve its full inhibitory potential, AT must bind a longer-chain heparin structure (a dodecasaccharide) [8]. In addition to inhibition of thrombin, heparin/AT formation also catalyzes the inhibition of blood coagulation Factors IXa, Xa, XIa, and XIIa [9].

Heparin possesses many other biological properties besides its anticoagulant activity; it inhibits smooth muscle cell proliferation [10], catalyzes lipoprotein lipase, binds to endothelial cells, inhibits the interaction of von Willebrand factor (vWF) with platelets [11], and in addition, interacts with a wide variety of biological proteins, including proinflammatory chemokines, growth factors, extracellular matrix proteins and leukocyte proteases, such as cathepsin G.

While the critical pentasaccharide sequence alone is sufficient for heparin to bind AT, we [12], and others [13], postulate that other, chemically unique heparin "unit structures" exist and that these unit structures mediate heparin's interaction with other proteins (Fig **1**). For example, we have shown that heparin binds to a unique domain of human von Willebrand factor, and that this unit structure within heparin remains chemically intact after purposeful destruction of the pentasaccharide unit structure. Similarly, we postulate that the unit structure that mediates heparin's ability to inhibit smooth muscle cell proliferation is distinct from its anticoagulant effects.

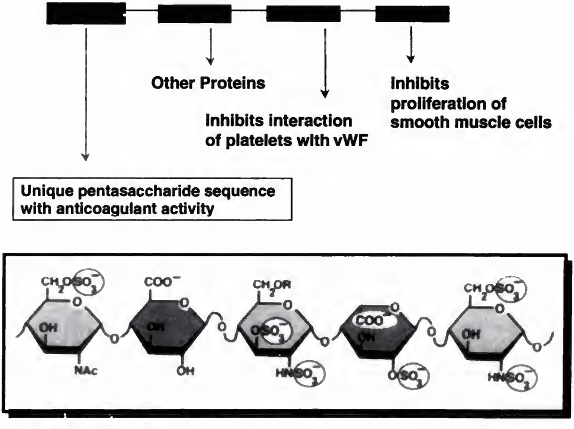
Other Proteins
Inhibits interaction of platelets with vWF
Inhibits proliferation of smooth muscle cells
Unique pentasaccharide sequence with anticoagulant activity
CH2OSO3-
COO-
CH2OR
CH2OSO3-
O
OH
OSO3-
COO
OH
NAc
OH
HNSO3-
OSO3-
HNSO3-

Fig. (1). Unit structures within heparin mediate its interaction with other proteins. We [12] and others [13] have proposed that unique arrangements of sulfated saccharides present within the heparin polymer chain form recognition elements for interaction with other proteins. For example, the pentasaccharide sequence [see ref. 19 and 20 and references cited therein] possesses anticoagulant activity due to interaction with AT, whereas a unique (but as yet undefined) saccharide sequence mediates interaction with von Willebrand factor or causes inhibition of smooth muscle cell proliferation.

Complexation with heparin induces a conformational change in many proteins including AT [14], fibroblast growth factor (FGF) [15], and mucous proteinase inhibitor [16]. Heparin is believed to interact with these proteins through formation of productive electrostatic interactions between the polyanionic groups of the glycosaminoglycan chain and the positively charged amino acids of the proteins. Synthetic peptides that encompass the heparin binding domains of vWF or AT have been synthesized, and these peptides compete quantitatively with the native proteins for heparin binding [17].

Molecular modeling analysis of heparin binding domain sequences of proteins and peptides in the data base shows that the spatial distribution of basic amino acids in these heparin binding sequences conforms to a motif wherein two basic residues, generally Arg, are separated by about 20 Å, facing on opposite sides of an α-helix or β-strand structure, and other cationic residues bridge the distance between these two Arg residues [18]. It is believed that heparin may form a coiled coil-like structure, wrapping itself around the peptide backbone. Such a complex might easily induce a change in protein/peptide conformation. We [19] and others [20] have shown by chemical synthesis and mutational replacement strategies, respectively, that particular cationic residues within AT are essential for recognition and binding of heparin at the high affinity site. Replacement or modification of these residues results in proteins (or peptides) which no longer bind heparin. We [19] suggested that AT Geneva, a naturally occurring mutant protein whose carriers display a predisposition toward thrombosis, is dysfunctional because an unfavorable distortion in the conformation assumed by the heparin binding domain sequence is caused by replacement of an essential Arg residue with Gln.

Clinical Uses of Heparin

Heparin-mediated anticoagulation is required in all cardiac surgery, and it is also commonly used in a variety of vascular surgical and catheterization procedures. Heparin is well entrenched as the standard treatment for producing surgical anticoagulation because its modality and therapeutic course are well characterized, and because heparin is used mostly in acute surgical situations. If need be, the anticoagulant effects of heparin can be allowed to wane over a prolonged period of time. Heparin is not absorbed by the gastrointestinal route and usual dosing is subcutaneous or intravenous [21]. Clinical benefits from anticoagulation with heparin treatment have been clearly shown in various studies. There are significant drawbacks associated with heparin therapy including lack of oral dosing, a narrow toxic-therapeutic window, occasional immune-related thrombocytopenia, spontaneous bleeding, and the inability to inhibit clot-bound thrombin. But overall, the major complication associated with heparin therapy is hemorrhage, occurring in as many as 10-15% cases [22]. Most clinicians believe that the bleeding complications arising from heparin therapy can be avoided if heparin anticoagulation is safely neutralized in a timely fashion. So far, these low molecular weight heparins are gaining acceptance as effective drugs for the prevention and treatment of venous thromboembolism, albeit with no clear evidence that they offer reduced bleeding complications. Low molecular weight heparin has so far not been widely adopted as an anticoagulant for arterial procedures, in part because of the longer duration of action, but mainly because there is no completely effective antidote. Protamine only incompletely reverses anticoagulation produced by low molecular weight heparins [23] because it does not adequately reverse the anti-factor Xa activity of the low molecular weight heparins [24].

Similarly, other anticoagulants in development or which have recently introduced into the market (such as platelet antagonists, thrombin inhibitors, defibrinogenating enzyme, and recombinant anti-clotting factors) will likely also be of limited usefulness because of the inability to reverse their effects and in addition, to the fact that most such agents will likely be very expensive to produce.

The Need for a Heparin Antagonist with Defined Activity

Thus, to use heparin optimally, its anticoagulant action must be precisely controlled in order to stop bleeding if necessary. Rapid reversal of heparin-induced anticoagulation is required after all cardiac surgical procedures and may also be needed following general vascular surgery. Protamine sulfate is the only agent currently available to reverse heparin-induced anticoagulation. It is a basic protein which contains an inordinate number of Arg residues. Protamine is isolated from fish sperm and its natural function is to bind to and protect DNA in aqueous environments. Protamine, which has been used clinically for 50 years, binds tightly to heparin forming multiple electrostatic contacts and a stable heparin/protein complex.

When bound to heparin, protamine completely neutralizes heparin's anticoagulant activity [25] by making heparin unavailable to potentiate the activity of antithrombin [26]. As might be expected, protamine indiscriminately also neutralizes all other biological effects of heparin, by overwhelming the anionic heparin with an abundance of positive charges.

Protamine treatment can produce a number of adverse cardiovascular and immunological effects that are potentially life-threatening. Protamine's high positive charge is thought to be responsible, in part, for many of the drug's toxic effects [27] including systemic vasodilation, bradycardia and signs of cardiac depression [28]. Since the severity of these systemic cardiovascular effects appears to be related to the dose of protamine and the rate of administration, standard clinical practice is to administer protamine as a relatively slow infusion [29]. Protamine can also produce pulmonary artery hypertension and pulmonary venoconstriction, leading to pulmonary edema and associated bronchoconstriction [30] and can produce allergic anaphylactic responses, mainly in individuals who have previously been exposed to protamine or to protamine-containing insulin products. Protamine can produce thrombocytopenia and leukopenia, and it is directly toxic to cardiac cells [31]; these adverse effects may be sequelae of the drug's action to activate complement and lead to the formation of cytotoxic inflammatory mediators [32]. There is also evidence that the use of protamine sulfate to reverse heparin anticoagulation during carotid endarterectomy increases the risk for postoperative stroke [33]. Fatalities from the use of protamine have been reported [34].

Recently, protamine has been shown to possess yet another potentially deleterious side effect. It is well known that endogenous and exogenous heparins inhibit DNA synthesis and subsequent growth of smooth muscle cells [35]. Exogenous and endogenous heparins may help suppress intimal hyperplastic lesions that result from smooth muscle cell proliferation following vascular injury (restenosis) [36]. Aside from suppressing smooth muscle cell migration and proliferation, heparin also alters the stability and activity of specific growth factors. Cell culture studies have shown that protamine exacerbates smooth muscle cell proliferation [37]. These results have also been confirmed in whole animal models. In both situations, protamine negated the beneficial antiproliferative effects of heparin. Therefore, there is increasing concern for the continued use of protamine because in the setting of vascular injury and repair, protamine administration may be especially harmful, leading to intimal hyperplasia, premature stenosis, and thrombosis [38].

Anesthesiologists and vascular surgeons clearly recognize these shortcomings of protamine sulfate. Despite its poor therapeutic index, protamine has been used since 1939, although with caution since there are no alternative means available to achieve heparin neutralization. Many methods, such as administration of a small or divided dose, have been instituted to try to prevent the adverse side effects of protamine. Indeed, high doses of heparin and of protamine neutralization are essential treatments in cardiac bypass procedures, which number approximately 700,000 annually in the United States alone. Protamine is used to neutralize heparin in approximately 800,000 general vascular surgeries or cardiac catheterization procedures per year in the United States. In the remaining general vascular procedures where concerns preclude the use of protamine, the anticoagulant effects of heparin are allowed to wane spontaneously over several hours under clinical monitoring.

Standards of surgical practice for the use of heparin and its reversal appear to be similar in the major global markets, while incidence of relevant cardiovascular diseases may vary somewhat among geographical regions. Still, it is estimated that as many as 4-5 million patients annually undergo intraoperative heparin reversal world-wide, nearly half of whom receive high dose reversal therapy for cardiac bypass procedures.

Candidate Heparin Antagonists Currently in Development

The need for a superior heparin antagonist, one with more selective biologic actions and an improved safety profile, has prompted new research efforts.

Recombinant Platelet Factor 4 (rPF4; Repligen Inc) was considered as a possible heparin antagonist and advanced through pre-clinical testing [39]. rPF4 was effective in reversing heparin anticoagulation in rats, but its use led to severe adverse reactions including anaphylaxis and acute pulmonary vasoconstriction and hypertension in some non-rodent species. Immune sensitization to heparin, when it complexes with rPF4, was also common, resulting in major morbidity and death. The research program at Repligen aimed at developing rRP4 has been suspended.

In another approach, the use of fragments of protamine prepared by proteolysis is being assessed as heparin antagonists. Whether these fragments, which tend to be of relatively high molecular weights, are any less toxic than protamine itself is currently under study. Poly-cationic protamine-like peptides bearing discrete positive charges have also been chemically synthesized. While there is a close relationship between cationic charge density and heparin-neutralizing efficacy, it is also evident that structural conformation of the antagonist,i.e. the three dimensional presentation of charged groups, is also a critical area of design. Without attention to structure, research that simply focuses on the net charge density of large protamine fragments is likely to produce compounds with a similar profile of side effects as protamine itself [27].

In yet another strategy, IBEX Pharmaceuticals is attempting to qualify recombinant heparinase I (Neutralase™) as a human therapeutic [40]. Neutralase is a short-duration drug which is rapidly cleared from circulation. Through hydrolysis of particular glycoside linkages of heparin, Neutralase reverses anticoagulation by enzymatically degrading heparin.

However, in initial phase III clinical trials, some patients receiving Neutralase developed abnormal bleeding complications which caused suspension of the trials. The reasons for these bleeding complications are not all together clear, but it may be that an incorrect dosing paradigm complicated by patient selection criteria and rigidity of the trial protocol mitigated against success of the trial. IBEX is currently in a revised phase III trial with Neutralase.

Another biological concern is what may be the effects of systemic administration of a heparinase enzyme? Endothelial and many other types of vascular cells display large quantities of heparin-like glycosaminoglycans on their surfaces, and these heparins are believed to play a supportive role in maintaining the antithrombotic phenotype of the vessel wall [56]. Stripping the vascular system of all its cell-surface glycosaminoglycans may not be healthy.

Yet another potential protamine replacement drug is currently undergoing evaluation. Recombinant human lactoferrin [41] is being produced in large quantities in cows' milk. Lactoferrin is a protein involved in host defense against infection and inflammation and interacts with heparin through an N-terminal domain of the protein which is enriched in cationic residues.

The dose of lactoferrin needed for reversal of anticoagulation is relatively high and the cost of production is not insignificant. Regardless, Gem Pharmaceuticals is pursuing recombinant lactoferrin as a protamine replacement.

Development of HepArrest

The CBI research program led to the development of a series of synthetic heparin antagonist peptides. Our research began with a fundamental analysis of how proteins interact with heparin. Based on the primary sequence of these domains, we initially derived, and then synthesized, a "consensus sequence" peptide which bound heparin with high affinity and specificity [17]. A feature of this consensus sequence is the interspersion of hydropathic residues within clusters of Arg and Lys residues, but the key to heparin binding was the correct "presentation" of a cationic surface which is complementary in space to the anionic charged surface of heparin [12,19,42].

Our first attempt in preparing a helix heparin-antagonist peptide, was a 19-residue peptide, termed Arg Helix #1 [42]. This peptide was predicted to assume a helix structure in solution in which the cationic residues of the peptide were suitably spaced so that their positive side chains would be oriented on the same side of the helix. The Arg Helix #1 peptide, succ-AEAAARAAARRAARRAAAR-amide, was found to be 75% α-helix by CD spectrometry and complexation with heparin increased helix content of the peptide to 100%. However, this peptide was not effective in reversing heparin induced anticoagulation in either factor Xa or aPTT biological assays. Based on iterative design concepts, a series of single copy cationic helix peptides were designed, synthesized, and tested [43,44]. All were effective to some degree in *in vitro* heparin-binding assays, but none were particularly effective in biological assays in reversing heparin-induced anticoagulation.

We next reasoned that we might more closely mimic the activity of protamine if we were to prepare a helix peptide that contained more than one copy of the binding sequence. In this scheme, two copies of Arg Helix #2 were synthesized individually but simultaneously on the α- and ε-amino groups of a C-terminal Lys residue, which had been first conjugated to the synthesis resin. The resulting peptide, designated Bis Arg Helix #2, has two helix #2 sequences joined $N \Rightarrow C \Rightarrow C \Rightarrow N$ through a common lysyl tether[43-45]. Bis Arg

Helix #2 bound heparin 3-fold better than Arg Helix #2, and in factor Xa neutralization assays, Bis-Arg Helix #2 showed a 3-fold better inhibition of heparin-AT binding than Arg Helix #2. In this assay, Bis Arg Helix #2 was only 2-fold poorer than protamine. Thus, by increasing the number of heparin-binding sequence within one compound we effectively increased the efficacy of the compound. The most exciting discovery was that Bis Arg Helix #2 was effective in reversing heparin-induced anticoagulation in the anesthetized guinea pig. Albeit at much larger doses than protamine, within one circulation time, Bis Arg Helix #2 completely reversed the anticoagulated state of the animal [43,45].

The structure of the helix heparin antagonist was progressively changed and optimized for heparin binding and biological efficacy. Compounds with three (Tris Arg Helix #3) and four (Tetra Arg Helix #3) copies of the helix arm were prepared, and tested for their ability to bind heparin and reverse anticoagulation in the anesthetized guinea pig [43,46,47]. The compounds were tested for their toxic effects on proliferating smooth muscle cells, and for their ability to antagonize heparin-induced inhibition of smooth muscle cell proliferation [43,48].

Finally, molecular modeling of Tris Arg Helix #3 docked with the anticoagulant pentasaccharide suggested that a constrained Tris Arg Helix #3 analog would favor multi-facial contacts with heparin, thus possibly making for a better heparin antagonist. Based on these results, a new heparin binding compound was designed, HepArrest^{TM}, wherein decreased flexibility in the tether region fixed the positioning of the helix peptide "arms" of the compound [46,49,50].

HepArrest is a viable alternative to Protamine

In designing HepArrest, we hoped to mimic the three-dimensional binding motif of the antithrombin-pentasaccharide complex whose crystal structure has recently been determined [51]. We postulated that if we could constrain two of the helical "arms" of Tris-Arg Helix #3 and allow the third "arm" to freely rotate we could attempt to mimic the "swinging-gate" AT model.

In the design concept of HepArrest, a β-turn mimetic provides the necessary constraints for creating a heparin binding "channel" within the Tris-Arg Helix #3 molecule Fig. (**2**). The β-turn is

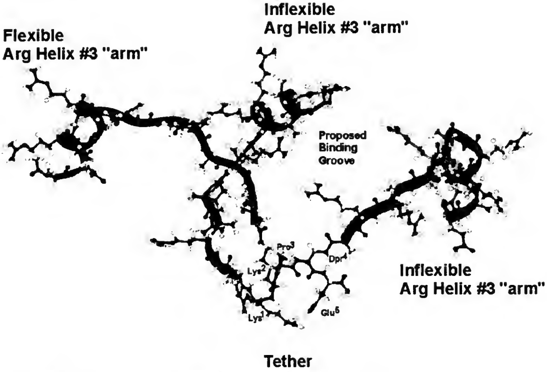

Fig. (2). Molecular model of the proposed structure of HepArrest. The α-amino group of Lys 1 and the ω-amino group of Dpr 4 (diaminopropionic acid) are conformationally constrained by Pro 3 in the tether region. As a result, the Arg Helix #3 sequences that originate at these amino groups from the proposed heparin pentasaccharide binding groove. Arg Helix #3 which extends from the ε-amino group of Lys 2 is spatially removed from the binding groove, and it is proposed to act as a "gate" which opens to accommodate heparin, then closes and locks-in the bound saccharide.

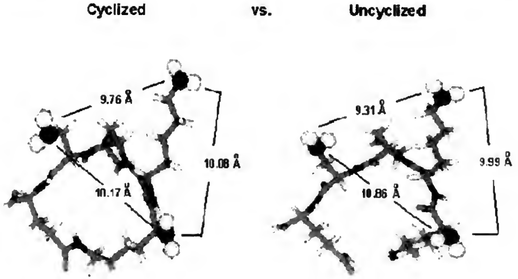

Fig. (3). Molecular model of the non-cyclized β-turn tether of HepArrest compared with a cyclized β-turn tether. Both structures were minimized using a dielectric constant of 3. The intramolecular distances (in angstroms) in the two possible structures are the same.

maintained by a proposed salt bridge between the side chains of Lys 1 and Glu 4 (Fig **3**). In HepArrest, we speculate that the Arg Helix #3 sequence extending from the ε-amino group of Lys2 will be free to form secondary contacts with heparin, when heparin is bound within the channel. We found through molecular modeling protocols that an uncyclized tether retains essentially the same low conformational freedom as a cyclized tether. Furthermore, modeling experiments in which the tether sequence was repeatedly minimized showed that a thermodynamically stable structure likely exists in which the intra-atomic distance and orientations of the side-chain amino groups in the uncyclized tether are no different than those in the cyclized tether Fig. (**3**). In constraining the tether region, the conformational freedom of the arms of HepArrest has been significantly reduced, thereby increasing the probability of a single three-dimensional heparin-binding conformation.

Synthesis of HepArrest

HepArrest has been synthesized by either conventional FMOC or tBOC chemistry protocols in high yield (≈ 93%) and purity (> 97%) as judged on the basis of analytical reverse-phase HPLC and quantitative amino acid analysis; mass spectral analysis by either ES/MS of MALDI protocols has proved unsuccessful due to the well documented problems in forming ions from highly cationic proteins and peptides.

In Vitro Biophysical Studies

The ability of HepArrest to bind heparin was assessed by isothermal titration calorimetry (ITC) and by CD spectrometry. The structural stability of HepArrest was assessed in thermal titration CD spectrometry and by differential scanning calorimetry (DSC).

ITC experiments were performed using protocols established at Commonwealth Biotechnologies [12,43,49,52] to assess binding between HepArrest and heparin. A 15 μM solution of HepArrest was titrated with 100μM heparin and saturation was achieved within the seventh injection of heparin Fig. (**4**). The isotherm was deconvoluted to yield the thermodynamic parameters for binding (Table 1) and the results show that the HepArrest binds heparin with the greatest potency (Kd 36 nM) as compared to any of the other helix peptides, and comparable in potency to binding of heparin by protamine (Kd 25 nM). HepArrest binds heparin 14-fold better than Tris Arg Helix #3.

Binding of HepArrest is enthalpically driven ($\Delta H = -35.4$ kcal/mol), resulting in thermodynamically stable complex between heparin and the peptide ($\Delta G = -10.3$ kcal/mol). In comparison, the ΔG of Tris-Arg Helix #3 was -8.74 kcal/mol, ($\Delta\Delta G = -2.01$ kcal/mol), showing that HepArrest forms a more stable complex. The stoichiometry of binding heparin is 2.3 peptide equivalents per heparin monomer, essentially the

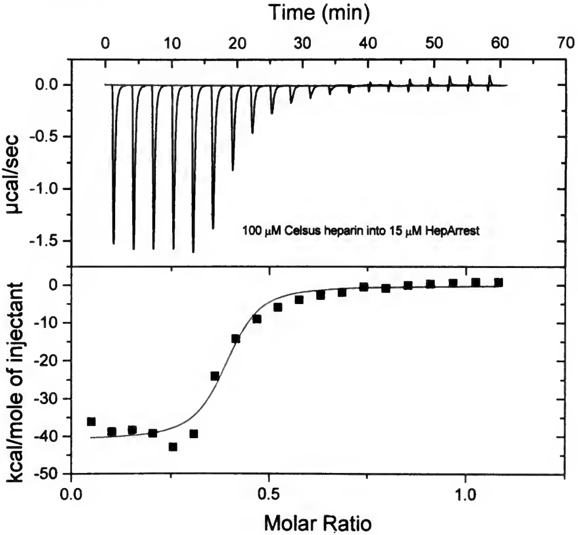

Fig. (4). ITC of the interaction between HepArrest and heparin. This representative binding isotherm shows the monotonic decrease in heat of binding as 10 µl aliquots of heparin are periodically titrated into a rapidly mixing solution of HepArrest. The Top Panel shows the instrument feedback (µcal/sec) necessary to maintain a constant temperature of 30 °C in the calorimeter cell after each injection of heparin. These results are then converted (Bottom Panel) to a binding isotherm. Saturation binding is achieved as the concentration of heparin in the calorimeter cell reaches a high molar ratio relative to the amount of peptide in the calorimeter cell.

same as that of Tris-Arg Helix #3, which we interpret as being consistent with the presence of multiple binding sites for HepArrest on each heparin monomer chain (15,000 molecular weight).

CD and DSC Analysis of HepArrest

Consistent with our previous findings [17,42,52], binding of heparin by HepArrest causes a shift in the CD spectral envelope of HepArrest, with a pronounced increase in α-helix character (data not shown). Binding of heparin affords about 8 kcal/mol thermal stability to the peptide.

Because of the rigid tether sequence in HepArrest, it was likely that this peptide would display a thermal unfolding event in the DSC even though few, if any, low molecular weight peptides possess sufficient structure to display a DSC thermogram. The DSC thermogram of HepArrest reveals a bell-shaped transition curve Fig. (**5**) which was readily fit by the integrated equation for a protein which unfolds with two transitions (two domains) in a two-state model. The presence of two transitions (Tm_1 35.5°C; Tm_2 43.1°C) shows that HepArrest contains two domains, one of which is less heat stable than the other. Perhaps the tether region forms the more stable domain and does not denature until a higher temperature is reached,

Table 1. Thermodynamic Parameters Which Mediate Binding Between Helix Peptide Family Members and Heparin[a]

Compound	Kd (μM)	ΔH (kcal/mol)	ΔS[b] (eu)	ΔG[c] (kcal/mol)	N[d]
Arg Helix #2	8.33	-62	-181	- 7.04	11.1
Bis Arg Helix #2	7.51	-36	- 95	- 7.10	9.1
Tris Arg Helix #3	0.51	-72	-209	- 8.74	2.7
HepArrest	0.036	-35	- 83	-10.3	2.3
Protamine	0.025	-37	- 78	-10.5	Not determined

[a] All experiments were done at 30°C in 50 mM phosphate buffer, pH 7.01 according to [17,19]. Generally, twenty 10 μl injections of 30 sec duration were made into rapidly mixing (400 rpm) peptide solution, with 2 min equilibration time between injections. For all experiments, the peptide was placed in the calorimeter cuvette, and heparin (Celsus USP grade) was placed in the dropping syringe. All isotherms are corrected by subtraction for the isotherm obtained following injection of heparin into buffer in the absence of binding peptide.

[b] $\Delta S = -(\Delta G - \Delta H)/T$

[c] $\Delta G = -RT\ln K$; 303°C

[d] The stoichiometry of binding, N, is expressed as the number of peptide equivalents per heparin monomer (15,000 molecular weight).

whereas the helix arms unfold with similar Tm's to those for Tris-Arg Helix #3. As predicted, binding of heparin stabilizes the structure of HepArrest, and obviates the low Tm domain; only a single Tm was observed (43.9°C).

Ex vivo Biological Assay of HepArrest

The biological competence of HepArrest was assessed by competitive factor Xa and aPTT assays. The competitive factor Xa neutralization assay and the modified aPTT assay measure the ability of a test agent to interfere with the interaction between heparin and AT, and are effective measures of the ability of a test agent to reverse heparin-induced anticoagulation [19,43,49]. The Factor Xa neutralization assay is a competitive binding assay based on the heparin assay of Teien which essentially measures heparin-antithrombin complex formation by its neutralization of Factor

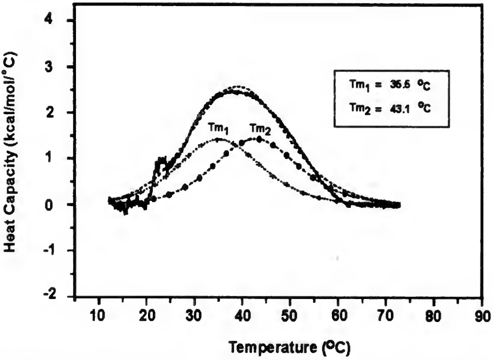

Fig. (5). DSC thermogram of HepArrest. The heat capacity change in a 100 μM solution of HepArrest in 30 mM KPO_4 buffer, pH 7.0, was monitored with respect to increasing temperature. The thermogram was corrected by subtraction of the thermogram obtained for buffer alone. The overall integrated area fit for the thermogram is shown , which was best fitted to a two-state transition but with two melting events (shown with their respective Tm values, Tm_1 and Tm_2).

Xa enzyme activity. Briefly, heparin (28 nM), purified human AT (280 nM) and test peptide (0-10 μM) are co-incubated at room temperature in the wells of a microtiter plate for 15 minutes. Factor Xa and a chromogenic substrate for Factor Xa are then sequentially added, and the residual activity of Factor Xa is measured colorimetrically. Binding of the test peptide to heparin diminishes the formation of heparin-antithrombin complex, and consequently, more residual Xa activity is observed. The degree of inhibition caused by the peptide is calculated as the percent reduction of heparin-antithrombin complex activity in the absence of peptide.

In the aPTT assay, a dose range of protamine or test agent is added to heparinized (0.15 units/ml) pooled, human plasma and the aPTT assay is performed as described using an automated fibrometer and thromboplastin reagent. The aPTT is a global measure of heparin's anti-thrombin effects, including catalysis of AT as well as direct heparin-thrombin interactions and the effects of heparin cofactor II.

As shown in Fig. (**6**), protamine and HepArrest are virtually indistinguishable in their abilities to compete with AT for binding heparin ($IC_{50} \approx 10$ nM), and there is a progressive increase in potency from the single copy helix compounds (exemplified by Arg Helix #2), to the multiple copy compounds (Bis Arg Helix #2, Tris and Tetra Arg Helix #3), to HepArrest. HepArrest is more than 10-fold more potent than the Tris Arg Helix #3 (IC_{50} 1580 nM). The minimum threshold dose of 0.6 μM of HepArrest causes observable reversal of anticoagulation.

Factor Xa Neutralization Assay

The ability of the helix family members to neutralize heparin mediated inhibition of Factor Xa activity is illustrated in Fig (**7**). In this assay, even Tris (and tetra) Arg Helix #3 and protamine are equipotent (IC_{50} 140 μM), and both compounds are 5-fold more potent than Bis-Arg Helix #2 and nearly 200-fold more potent than Arg Helix #3, the single copy helix peptide of which Tris-Arg Helix #3 is composed. Although HepArrest itself has not been fully tested in this assay, we can safely predict that it will be at least as potent as Tris Arg Helix #3 and quite likely equipotent with protamine.

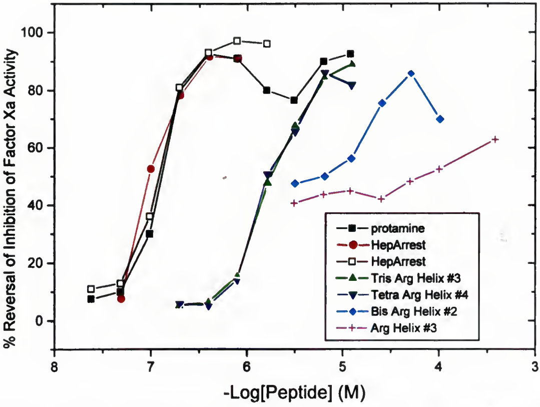

Fig. (6). Reversal of heparin-induced anticoagulation by the helix peptides as assessed by aPTT assay. Increasing concentrations of protamine or test agent were added to heparinized (0.15 units/ml) pooled, human plasma and the aPTT (clotting time) measured. Protamine and HepArrest are equipotent in reversing anticoagulation and HepArrest is more potent than any of the other helix peptide family members.

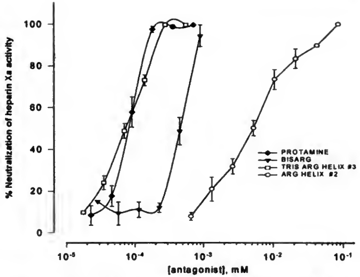

Fig. (7). Factor Xa inhibition caused by AT in the presence of heparin and in the presence of different helix peptides. As the concentration of protamine or test peptide is increased (in the range of 0-10 μM), less heparin is available to potentiate AT inhibition of Factor Xa proteolytic activity and so more residual Factor Xa activity is measured colorimetrically. As shown, protamine and Tris Arg Helix #3 are equipotent in this assay, and both are significantly more potent than the other helix peptide family members.

Binding of Low Molecular Weight Heparins

As shown (Fig **8**), Enoxaparin™ (Rhone Poulenc Rorher), Fragmin™ (Upjohn/Pharmacia), and Normiflo ™ (Wyeth Ayerst) all cause prolongation of the thrombin clotting time (TCT), albeit at different concentrations and to different levels. Although the profile of inhibitory activity of LMW heparins favors their inhibition of Factor Xa

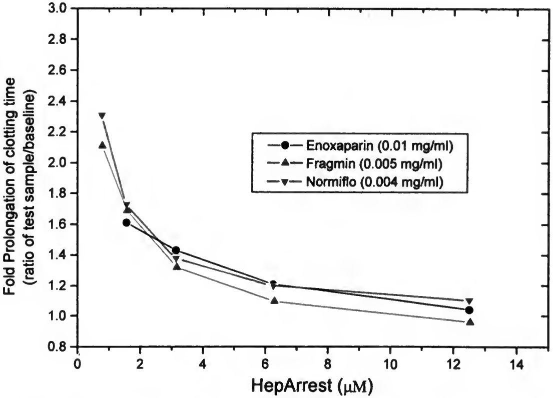

Fig. (8). Reversal of anticoagulation mediated by low molecular weight heparins as assessed by thrombin clotting time assay. Increasing concentrations of HepArrest were added to human plasma which contained the indicated concentration of Enoxaparin, Fragmin, or Normiflo. The ordinate is expressed as the fold prolongation of clotting time over baseline values.

over Factor IIa (thrombin), low molecular weight heparins still retain significant thrombin-inhibiting activity at higher doses. HepArrest is highly effective at reversing the anti-thrombin effects of these low molecular weight heparins, with IC 50 values in the low µM range. It is thought that hemorrhagic complications from the use of low molecular weight heparins may arise more from their anti-IIa action than from their anti-Xa action. Accordingly, HepArrest may prove to be an effective antidote for bleeding associated with low molecular weight heparin therapy.

HepArrest is also effective in neutralizing Factor Xa inhibition caused by Normiflo, Enoxaparin, and Fragmin (Fig **9**). Here, we observed a clear-cut dose dependence for reversal inhibition of Factor Xa activity mediated by the low molecular weight heparins. The ordinate values are expressed as the percent neutralization of anti-Factor Xa activity.

Other *Ex vivo* Experiments

Smooth muscle cells produce heparin-like compounds that are growth inhibitory and these heparin-like compounds likely play a regulatory role in maintenance of vascular tone that is perturbed at sites of vascular injury [36,37]. Cultured smooth muscle cells also respond to added doses of heparin, which causes a marked inhibition of proliferation [35,36].

Thus, the effect of test peptides and protamine on smooth muscle cell proliferation was measured on vascular smooth muscle cells. Primary cultures of smooth muscle cells were prepared from bovine aorta essentially as described by Edelman *et al.*, [37].

In comparison with cells grown in media alone, protamine alone causes dose related cell death of cultured smooth muscle cells, evident at concentrations as low as 0.1 mg/ml (data not shown). All of the helix peptides, at concentrations as high as 1.5 mg/ml, were non-toxic to the cells (data not shown).

Heparin inhibits the proliferation of smooth muscle cells in a dose dependent fashion and this effect is more pronounced with increasing time of incubation. Of all the agents tested, only protamine (50 µg/ml) completely reversed heparin-induced inhibition of cell growth (data not shown). None of the helix peptides tested, released cell growth from

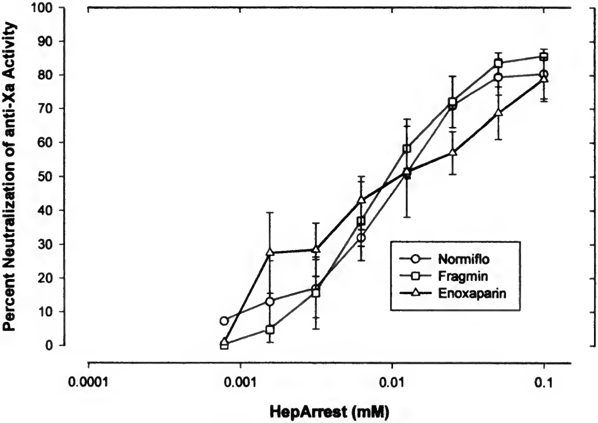

Fig. (9). Reversal of anticoagulation mediated by low molecular weight heparins as assessed by Factor Xa enzyme assay. The effect of increasing concentrations of HepArrest on Factor Xa activity in the presence of the indicated amounts of the LMW heparins was assessed by chromogenic enzyme assay.

heparin inhibition. It appears from these and other experimental results that the heparin unit structure which mediates inhibition of smooth muscle cell proliferation recognizes a β-strand presentation of a cationic surface, and *not* a helix presentation of a cationic binding surface. Hence, none of the helix peptides bind the heparin region responsible for inhibition of smooth muscle cell proliferation, which suggests that the helix family members would *not* negate this beneficial effect of heparin.

In vivo Studies - Efficacy Studies in the Heparinized, Anesthetized Guinea Pig

Heparinized, anesthetized guinea pigs are used to assess the efficacy of the helix family compounds in reversing heparin-induced anticoagulation. In this model, the anesthetized animal is given a sufficient dose of heparin (35 or 150 units/kg) necessary to significantly elevate the clotting time over baseline values. Heparin administration is followed 5 or 15 minutes later by intravenous administration of test agent, either given as a bolus dose or in some experiments as discrete incremental administrations of lower doses. The ACT (activated clotting time, measured by injecting freshly drawn blood into celite-containing tubes and recording time required for coagulation) and the aPTT are then measured over the course of the experiment.

Whether given as a single bolus dose (Fig **10**) or administered as injections of discrete doses (Fig **11**), HepArrest causes an immediate reversal of heparin-induced anticoagulation within one circulation time as measured by either the ACT or aPTT assays. In the absence of administered HepArrest, the clotting time remains prolonged, and even at 65 minutes post heparin infusion the aPTT time is still nearly 2.5 fold higher than baseline levels (Fig **10**). HepArrest and protamine are both effective in reversing heparin-induced anticoagulation in the guinea pig.

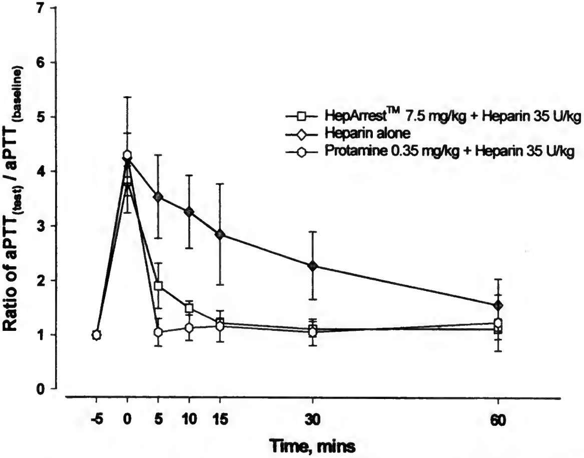

Fig. (10). Bolus infusion of HepArrest or protamine causes reversal of heparin-induced anticoagulation in the anesthetized guinea pig. At time –5 minutes, heparin was administered, resulting in a 4 fold increase in aPTT which slowly waned over the course of the experiment. Even at 65 minutes post infusion, however, the aPTT was still 2-fold elevated over baseline levels. Administration of HepArrest or protamine causes an immediate restoration of clotting time within one circulation time.

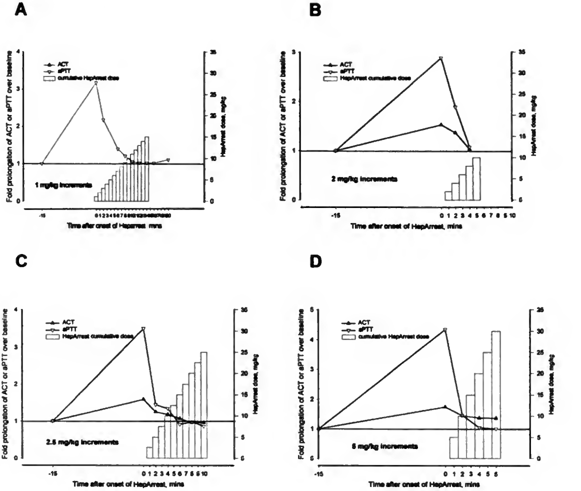

Fig. (11). Infusion of HepArrest causes restoration of normal coagulation in the heparinized, anesthetized guinea pig. HepArrest was administered using four different dosing regimens to guinea pigs treated with 150 units/kg of heparin at 15 minutes before test drug administration. In the first experiment (A), an animal received a series of incremental doses of 1.0 mg/kg for a total cumulative dose of 15 mg/kg. A second animal (B)received incremental injections of 2.0 mg/kg once a minute for 5 minutes. A third animal (C) received a total cumulative dose of 25 mg/kg as a result of 10 incremental injections of 2.5 mg/kg given once a minute, and the fourth animal (D) received a total dose of 30 mg/kg over 6 minutes given as incremental doses of 5 mg/kg. As measured by the aPTT or ACT assays, heparin caused a significant increase in clotting time, reaching as much as 5-fold elevation over baseline values. As little as 4 mg /kg of HepArrest restored normal clotting time, and cumulative doses up to 30 mg/kg did not cause anticoagulation.

In Vivo Studies in Yorkshire Pigs

In Yorkshire pigs, protamine treatment is known to produce the adverse effects of pulmonary artery hypertension and venoconstriction [53]. In these studies, pigs are anesthetized and two baseline measurements of heart rate, systemic arterial pressure, pulmonary arterial pressure, and cardiac output are made. Blood samples are also collected for determination of whole blood aPTT and ACT time. Heparin(150 units/kg) is then given intravenously approximately 15-25 minutes prior to administration of test drugs. A third set of measurements is then made immediately before administration of a test dose of protamine or HepArrest.

One group of pigs received a single intravenous dose of protamine sulfate (2 mg/kg, administered as a rapid, bolus injection). A second group of animals was treated with 4 rapid bolus intravenous doses of HepArrest (10 mg/kg per dose) at 5 minute intervals. Measurement of all cardiovascular parameters and blood sample collections were performed at 5, 10, 15, 30 and 60 minutes after the initial dose of test compound.

In these studies [49,54], heparin caused a near 3-fold elevation in clotting time (aPTT or ACT assays). The dosing regimens of protamine and HepArrest totally neutralized heparin-induced anticoagulation within 10 minutes after administration (Fig 12). In still another experiment

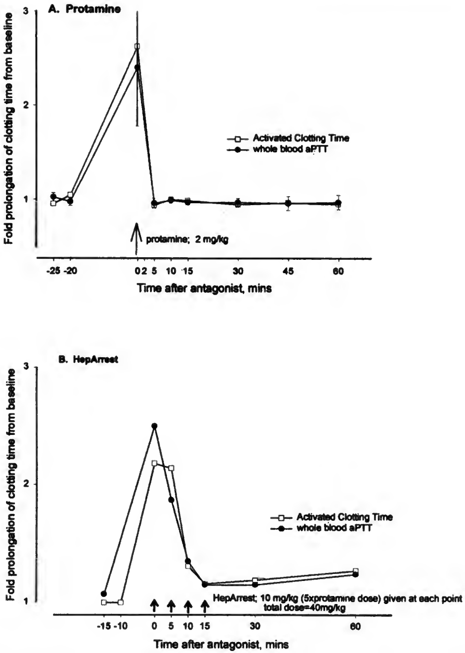

Fig. (12). HepArrest and protamine causes reversal of heparin-induced anticoagulation in the Yorkshire Pig. Pigs were treated with 150 units heparin per kg at 15-20 minutes prior to administration of protamine (top Panel; 2 mg/kg) or HepArrest (bottom panel; 4 x 10 mg/kg at 5 minute intervals). In these experiments, HepArrest was intentionally administered at suprapharmacological doses, in order to maximize any deleterious side effects of the drug. Heparin caused a near 3-fold elevation in clotting time (aPTT or ACT assays) and the dosing regimen of protamine or HepArrest totally neutralized heparin-induced anticoagulation within 10 minutes after administration.

in the heparinized (150 units/kg) pig, infusion of 4-6 mg/kg of HepArrest restored coagulation and full reversal of coagulation to baseline levels was produced with 10 mg/kg (Fig **13**).

Of critical importance in these studies was the evaluation of the propensity of protamine and HepArrest to produce acute adverse cardiovascular effects. A variety of hemodynamic parameters including systemic arterial pressure, pulmonary arterial pressure, cardiac output and heart rate were thus monitored.

Protamine administration caused an immediate elevation in pulmonary artery pressure (Fig **14A**), whereas HepArrest administration at 20 times the

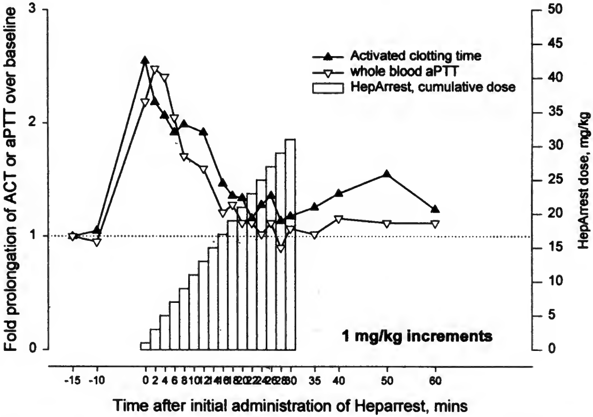

Fig. (13). Infusion of pharmacological doses of HepArrest causes restoration of normal coagulation in the Yorkshire Pig. HepArrest was administered to a 21 kg pig at incremental doses of 1 mg/kg/min following systemic heparinization with 150 units/kg. Correction of coagulation was observed upon administration of between 4 and 6 mg/kg of HepArrest, with full restoration to base line coagulation at 10 mg/kg. Note that after administration of 50 mg/kg of HepArrest, there was a slight rebound anticoagulation effect, but no adverse hematological effects were observed even after administration of as much as 60 mg/kg of HepArrest.

dose of protamine caused no significant changes in pulmonary artery systolic or diastolic pressures (Fig **14B**). Protamine caused a reduction in heart rate of about 10% for 15 minutes after dosing and a transient increase of systemic arterial pressure evident at 2 minutes after dosing, followed by a more pronounced fall of arterial pressure of 15-20 mm Hg that returned to normal or somewhat above normal by 15 minutes (data not shown). In contrast, HepArrest caused a only a moderate reduction in heart rate of 5-10% but had no adverse affect on arterial systemic pressure (data not shown).

Protamine also produced sustained depression of cardiac output by approximately 20% for the entire 60 minute observation period (Fig **15A**), whereas HepArrest showed only a moderate affect on cardiac output(Fig **15B**).

In Vivo Studies in Dogs

Guinea pigs are useful for dose finding experiments, and pigs are useful models to assess the effect of the test agents on blood pressure variables, but one of the more pronounced adverse affects of protamine therapy is systemic vasodepression [55]. This perturbation cannot be observed in the pig, and so dog trials were done to assess the efficacy of HepArrest in pharmacological doses in the canine and to assess the effect of HepArrest on canine hemodynamics, with a view towards its effect on vasodepression.

As shown (Fig **16**), protamine causes an immediate and sustained depression in mean cardiac output, reaching a peak depression of almost 45% within the first two minutes of administration. The dose of protamine (1.95 mg/kg) was 1.3 times the neutralizing dose for heparin (150 units/kg given at −10 minutes). This corresponds to a typical neutralizing dose. Note that following protamine treatment, mean cardiac output never recovers to baseline values, and at 60 minutes post protamine treatment, the cardiac output is still depressed by nearly 20%.

These results are contrasted with those obtained by treating the animal with HepArrest infusion

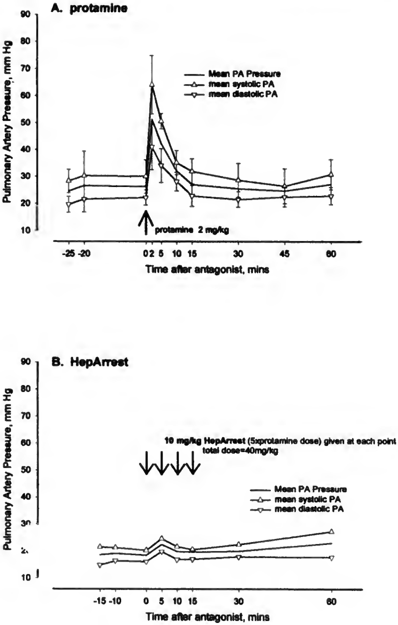

Fig. (14). Effect of high doses of HepArrest or pharmacological doses of protamine on pulmonary artery hypertension in the heparinized Yorkshire Pig. Pigs were treated with 150 units/kg of heparin at 15-20 minutes prior to administration of 2 mg/kg of protamine (top panel) or 4 doses of HepArrest (10 mg/kg) given at 5 minute intervals (bottom panel). Protamine administration caused an immediate elevation in pulmonary artery pressure whereas HepArrest administration at 20 times the dose of protamine caused no significant changes in pulmonary artery systolic or diastolic pressures.

(11.3 mg/kg total dose; Fig **16**). Here, there was a modest increase in cardiac output (to 21% above baseline) with return to baseline values at about 40 minutes post treatment. Several dogs have now been done at varying doses and administration routes with essentially the same results; a transient effect on cardiac output with short-term recovery to baseline values.

A drop in cardiac output (due to vasodilation) leads to systemic hypotension. As shown (Fig **17**), treatment with protamine leads to an immediate, severe drop of nearly 60% in arterial blood pressure which only slowly recovers to baseline at about 50 minutes post treatment. Even at this time, however, the cardiac output in the protamine-treated animal is still depressed by about 20%.

Again, contrast these results with those obtained by treating the animal with HepArrest (Fig **17**). There is a modest increase in blood pressure with return to baseline values at 40 minutes. In all dogs

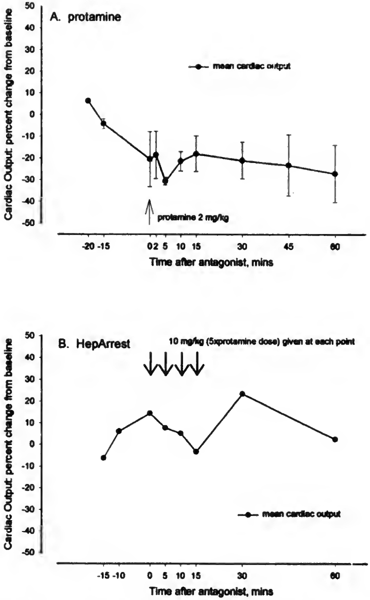

Fig. (15). Effect of high doses of HepArrest or pharmacological doses of protamine on cardiac output in the heparinized Yorkshire Pig. Pigs were treated with 150 units/kg of heparin at 15-20 minutes prior to administration of 2 mg/kg of protamine (top panel) or 4 doses of 10 mg/kg of HepArrest at 5 minute intervals (bottom panel). Protamine administration produced a sustained depression of cardiac output by approximately 20% for the entire 60 minute observation period (upper panel), whereas HepArrest, even at the suprapharmacological dose used, did not compromise cardiac output (bottom panel).

examine, the effect is qualitatively the same; there is a transient effect on blood pressure with return to normal baseline values.

Both agents are effective for reversing heparin-induced anticoagulation (data not shown); Protamine or HepArrest restore coagulation values within 15 minutes of their administration.

CONCLUSIONS; IMPLICATIONS

Virtually all invasive vascular surgeries require the use of heparin. In procedures performed which use high doses of heparin, protamine reversal is always used. Such procedures include coronary artery bypass grafts, cardiac valve repair, heart transplant and repair of congenital heart problems. In still other general vascular surgical procedures, heparin is used routinely and is commonly, although not always, reversed with protamine. These latter procedures include aortic aneurysm repair, AV fistula grafts for dialysis, carotid endarterectomy, complex vascular reconstructions and femoral popliteal bypass.

Currently, there are no options except protamine in clinical use for heparin reversal in the United States. Although alternative agents are either in

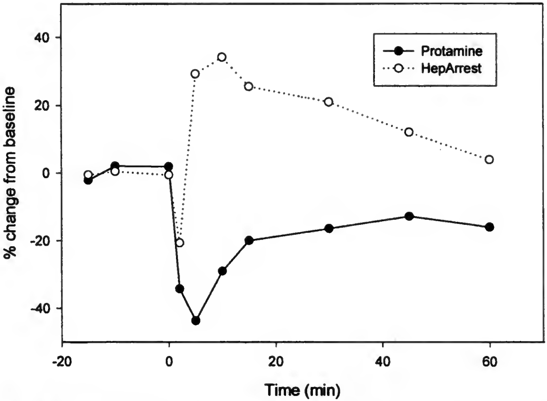

Fig. (16). Effect of protamine or HepArrest on mean cardiac output in the dog. At −10 min, anesthetized dogs were administered 150 units heparin/kg. At zero time, dogs were either administered 1.95 mg protamine/kg, or 2.5 mg HepArrest (bolus injection), followed by 0.25 mg/kg/min HepArrest (15 min), followed by 0.5 mg HepArrest/kg/min (10 min) (total dose 11.5 mg/kg). Protamine-treated animals (N=2) showed an immediate, sustained and severe drop in mean cardiac output (expressed as the percent change from baseline values). Dogs (N=10) treated with HepArrest (at varying doses up to 2 mg/kg/min x 30 min) showed a modest increase in cardiac output followed by a recovery to baseline.

development or in clinical trials, rPF4 is no longer under clinical consideration and the use of Neutralase is now being reassessed. Regardless, there is a strong rationale to find an effective and safe alternative to protamine.

The consensus opinion of surgeons interviewed during a marketing survey commissioned by Commonwealth Biotechnologies was that protamine is difficult to handle and titrate, but is effective once you have learned how to use it. Further, protamine was considered potent enough, but it could not be given as a bolus to achieve a very rapid onset of effect. Virtually all physicians interviewed felt that a superior replacement of protamine would be most useful and that a new drug for heparin reversal would be welcome.

Thus, surgeons and anesthesiologists that use protamine do so knowing that the drug is toxic and use it only because there is no safe alternative. Reversal of heparin-induced anticoagulation by protamine at the end of a surgical procedure is often accompanied by hemodynamic problems caused by protamine at the time when patients are most unstable and vulnerable. Unpredictable life-threatening anaphylactic responses can occur while reversing heparin induced anticoagulation and although the percentage of severe adverse effects is small, fatalities still occur from protamine use.

HepArrest is a superior heparin antagonist and has been designed to be highly effective and substantially safer than protamine. HepArrest has comparable potency to protamine for reversing heparin-induced inhibition of blood coagulation, and is able to neutralize anticoagulation caused by low molecular weight heparins. *In vivo* studies confirm the ability of HepArrest to effectively reverse heparin-induced anticoagulation without producing any of the acute adverse hemodynamic or cytotoxic effects exhibited by protamine. The apparent safety advantages for HepArrest include reduced risk for acute hemodynamic adverse effects. These highly promising pharmacological properties predict that HepArrest will fill a substantial unmet need for vascular surgeons and cardiac anesthesiologists who perform coronary

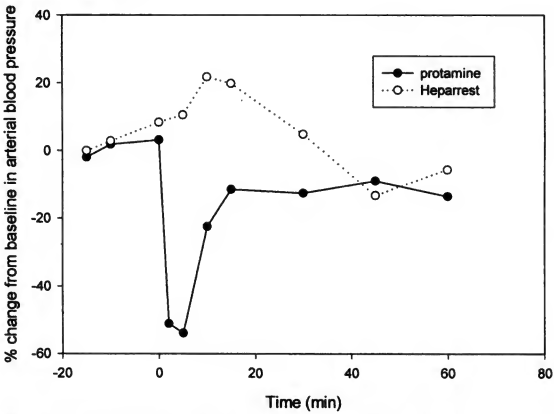

Fig. (17). Effect of Protamine or HepArrest on arterial blood pressure in the dog. At −10 min, anesthetized dogs were administered 150 units heparin/kg. At zero time, dogs were either administered 1.95 mg protamine/kg, or 2.5 mg HepArrest (bolus injection), followed by 0.25 mg/kg/min HepArrest (15 min), followed by 0.5 mg HepArrest/kg/min (10 min) (total dose 11.5 mg/kg). Protamine-treated animals (N=2) showed an immediate severe drop in blood pressure (expressed as the percent change from baseline values). Dogs(N=10) treated with HepArrest (at varying doses up to 2 mg/kg/min x 30 min) showed only a modest increase in mean arterial blood pressure followed by recovery to baseline.

artery bypass grafts and several other major vascular surgical procedures.

ABBREVIATIONS USED

ACT = Whole blood activate clotting time assay

aPTT = activated partial thromboplastin time assay

AT = Antithrombin

CD = Circular dichroism

HPLC = High performance liquid chromatography

ITC = Isothermal titration calorimetry; succ, succinyl

TCT = Thrombin clotting time assay

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The Role of Interferon-Alpha in the Treatment of Myeloproliferative Disorders

Claudia Fiorani, Stefania Tonelli, Barbara Casolari and Stefano Sacchi*

Dipartimento di Scienze Mediche Oncologiche e Radiologiche, Sezione di Medicina Interna, Oncologia ed Ematologia, Policlinico – Via del Pozzo, 71 – 41100 Modena, Italy

Abstract: The interferons are cytokines with a wide array of biological properties. In hematological malignancies the most used IFN class is -α; it has been used for thirty years but the mode of action is still not absolutely clear. Nevertheless, the benefits of IFN-α for the treatment of CMD have been described in particular for CML and less for PV, ET and MMM.

IFN-α is presently considered the golden standard of therapy for CML patients not eligible for SCT; the antileukemic effect has been well documented by hematological and cytogenetic response. The survival advantage for IFN treated patients is remarkable in comparison with patients treated with conventional chemotherapy. Recently, the combination IFN-α plus Ara-C has demonstrated to increase the rate of major cytogenetic response and to prolong survival.

To date, there is not a generally accepted treatment for ET, PV and MMM, which can reduce the risk of thromboembolism and/or hemorragic events. In several subsets of ET and PV patients, IFN-α can be considered as first line therapy.

IFN-α is usually associated with the development of early and later side effects, that reduce the enthusiasm for its use. In the future PEG-IFN-α would improve the quality of life of IFN-treated CMD patients.

INTRODUCTION

The interferons (IFNs) are cytokines with a wide array of biological properties, which include viral, immunomodulatory, antiproliferative and differenziative effects. They are designated as type I and type II IFN [1]. The former consist of four major classes IFN- α,- β,- ω,- τ, whereas the latter only of IFN- γ. Type I IFN are encoded in a gene cluster on human chromosome 9 [2]. They are produced by leukocytes and fibroblasts. The type I IFN family members are involved in a variety of activities: antiviral, antiproliferative and immunomodulatory. The most important are: stimulation of cytotoxic activity of lymphocytes, natural killer cells and macrophages; induction of MHC class I structures and other surface markers; activation of the Janus Family Kinases (Jak)-STAT signal transduction pathway [3, 4].

IFN-γ is produced and released by lymphocytes (CD4 TH1, CD8 and NK cells) upon activation with mitogens. IFN-γ causes differentiation of T lymphocytes, proliferation and differentiation of B lymphocytes, stimulation of MHC class II antigen expression, induction of B cell immunoglobulin production, activation of monocytes and macrophages, induction of IL 2 receptors [5].

It thus appears that IFN- α - β, -γ probably have distinct physiological functions. IFN- α and -β may preferentially act as antiviral agents; IFN-γ as an immunomodulator, IFN-α as an antineoplastic agent. The functions of IFN are mediated through interaction with receptors; IFN type I share the same receptor complex, whereas IFN-γ binds to a distinct receptor [6,7]. The binding to receptors leads to the activation of signal transduction pathways that stimulate the transcription of a defined genes; the final result is the production of proteins that are the not well known mediators of IFN biological activities. IFN can inhibit the replication of several cell types, such as endothelial, myeloid, lymphoid, mammary, epithelial and liver cells, but the antiproliferative mechanism is still

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*Address correspondence to this author at the Dipartimento di Scienze Mediche Oncologiche e Radiologiche, Sezione di Medicina Interna, Oncologia ed Ematologia, Policlinico – Via del Pozzo, 71 – 41100 Modena, Italy; Tel. No.: +39-059-422175; Fax No.: +39-059-424549; E-Mail: ssacchi@unimo.it

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largely unknown. In hematological malignancies the most used IFN class is -α; it has been used for thirty years but the mode of action is still not absolutely clear. Nevertheless, the benefits of IFN-α for the treatment of myeloproliferative disorders have been described in particular for chronic myeloid leukemia (CML) and less for polycythemia vera (PV), essential thrombocythemia (ET) and myelofibrosis with myeloid metaplasia (MMM).

CML, PV, ET and MMM were grouped for the first time in 1951 by Dameshek [8] under the common heading of chronic myeloproliferative disorders (CMD). These conditions are characterized by a neoplastic proliferation of the multipotent stem cell giving rise to the production of one or more myeloid lineages. A common characteristic is a chronic clinical evolution and a programmed but extremely variable tendency towards a terminal blast crisis, inevitable in CML, possible in PV, ET, and MMM. In the therapy of CML, remarkable advances have been made and IFN-α is presently considered the golden standard treatment for patients not eligible for allogenic stem cell transplantation (SCT). In contrast little progress has been made in the treatment of ET, PV and MMM; to date there is not a generally accepted treatment for these diseases which can reduce the risk of thromboembolism and/or hemorragic events.

CHRONIC MYELOID LEUKEMIA

CML accounts for 7% to 15% of all leukemias in adults, with an annual incidence of 1 to 1.5/100,000 population [9]. There is a male predominance (male:female ratio 1.4 -2.2 to 1). The peak incidence is in the fifth and sixth decades of life [10]. The etiology of CML is unknown; it is an acquired disorder. In some cases there is correlation with the HLA antigens CW3 and CW4 [11]. A higher incidence of CML was reported among survivors of the atomic bomb explosions at Hiroshima and Nagasaki, in groups of patients who underwent radiotherapy for ankylosing spondylitis and uterine cervical cancer [12,13]. Toxic chemicals or viruses have not been identified as a risk for CML.

CML is characterized by abnormal increased proliferation of a myeloid population showing the Philadelphia (Ph) chromosome, which is the result of a reciprocal translocation between the long arms of chromosomes 9 and 22 [14-17]. The classical 9;22 translocation is present in about 90% of cases of CML; variant cytogenetic changes are present in about 5% of cases and involve chromosomes 9, 22 and a third chromosome [18]. The Ph translocation results in the fusion of the bcr and abl genes; this hybrid bcr-abl oncogene produces an abnormal RNA transcript that encodes for a 210 kdal (P210) fusion protein showing tyrosine kinase activity. Less than 10% of CML patients lack the Ph chromosome [19]. In 35% of patients with CML Ph-, it is possible to identify bcr-abl rearrangement and these patients have a clinical course and prognosis similar to that of Ph+ patients. The patients who are Ph and bcr/abl negative respond less to therapy and have a shorter survival time than patients Ph+.

Clinical Manifestations

CML usually has a biphasic or triphasic course. Standard definitions of three phases have been proposed (Table 1). In the chronic phase, CML is asymptomatic from 15% to about 45% of all cases. Symptomatic patients suffer from anorexia, weight loss, fatigue, early satiety for splenomegaly and increased sweating. The main physical sign at diagnosis is splenomegaly (50%); hepatomegaly is less common (10% to 40% of patients). In the accelerated phase some patients may be asymptomatic, others may have fever and night sweats or progressive enlargement of the spleen.

In the blastic phase patients are more likely to have symptoms such as weight loss, fever, night sweats and bone pains. Symptoms of anemia, infectious complications and bleeding are common in this stage. Signs of central nervous system leukemia, may be seen in particular with lymphoid blastic transformation. Some CML patients develop a blastic phase without evidence of an accelerated phase.

Prognostic Features

The determination of risk factors for the prediction of survival time at diagnosis has been of great interest [20]. Several studies have identified a number of poor prognostic features; they can be patient, disease or treatment associated (Table 2). Single risk factors are of only limited prognostic value, but their value is increased when combined together. Several prognostic scores were proposed [21]. A multivariate analysis of prognostic factors has been used by Sokal to identify characteristics with prognostic significance [22]. He considered four variables (age, spleen size, platelet number, percentage of peripheral blasts) using a Cox proportional hazard model and classified patients

Table 1. Definitions of Chronic, Accelerated, and Blastic Phase of CML

Chronic phase CML	
Early	Equal or less than 12 months from the diagnosis
Late	More than 12 months from the diagnosis
Accelerated phase CML	Multivariate analysis-derived criteria
	Peripheral blasts 15% or more
	Peripheral blasts plus promyelocytes 30% or more
	Peripheral basophils 20% or more
	Thrombocytopenia $< 100 \times 10^9/L$ unrelated to therapy
	Cytogenetic clonal evolution
	Other criteria used in common practice
	Increasing drug dosage requirement
	Splenomegaly unresponsive to therapy
	Marrow reticulin or collagen fibrosis
	Marrow or peripheral blasts $\geq 10\%$
	Marrow or peripheral basophils ± eosinophils $\geq 10\%$
	Triad of WBC $> 50 \times 10^9/L$, hematocrit $< 25\%$, and platelets $< 100 \times 10^9/L$ not controlled with therapy
	Persistent unexplained fever or bone pain
Blastic phase CML	30% or more blasts in the marrow or peripheral blood
	Extramedullary disease with localized immature blasts

into three groups (low, intermediate and high risk). Poor prognosis was associated with older age, high platelet count or blast percentage and large spleen size. The hazard ratio was less than 0.8 for low risk patients, between 0.8 and 1.2 for intermediate risk and more than 1.2 for high risk. This index has two limitations: it is calculated by a complicated mathematical formula and was developed from patients treated with conventional chemotherapy; therefore it could be less predictive for patients treated with IFN-α.

Kantarjian has proposed other staging systems [23,24]. He identified the overall and the clinical models both based on values of hazard ratios, derived from solving a mathematical equation, as the Sokal proposed model. The first, using a multivariate analysis, identified five independent poor prognostic factors: age ≥ 60 years, black race, marrow basophils $\geq 3\%$, blood basophils $\geq 7\%$ and cytogenetic clonal evolution. The second model was based on the same factors, excluding the bone marrow findings and including two other independent poor prognostic factors: platelet count $\geq 700 \times 10^9/L$ or of less than $150 \times 10^9/L$ and increasing splenomegaly.

The synthesis prognostic model is otherwise based on a simple enumeration of the number of poor prognostic factors present at diagnosis [24]. Patients are categorized as having stage 1, 2 or 3 disease if they have none or one (stage 1), two (stage 2) or three or more (stage 3) of the following poor prognostic factors: 1) age ≥ 60 years, 2) peripheral blasts $\geq 3\%$ or bone marrow blasts $\geq 5\%$, 3) peripheral basophils $\geq 7\%$ or marrow basophils $\geq 3\%$, 4) platelet count $\geq 700 \times 10^9/L$, and 5) splenomegaly ≥ 10 cm below the costal margin.

Several groups [25,26] have reported that the Sokal score is a poor discriminator for survival of patients treated with IFN.

Hasford *et al.* [26] found that the synthesis model did not separate survival curves well when applied on a cohort of German patients. As a consequence they developed a new prognostic scoring system for patients treated with IFN that distinguishes three risk groups, both in learning and in validation samples. They collected data on 1573 patients from the most current IFN studies in CML world wide and 1303 patients were eligible for inclusion in this analysis. They obtained the new prognostic score with a different formula which weighted age, spleen size, percentage of peripheral blasts, eosinophils, basophils and platelet count. The prognostic relevance of the two additional factors (peripheral eosinophils and basophils) had been previously recognized in low-number-patient studies. The new scoring system allows a good discrimination of survival in three different groups: low, intermediate and high risk and it has been validated in several studies with a retrospective meta-analysis. To date, however the most used staging systems remain the Sokal and synthesis models.

Since IFN-α treatment is expensive and the quality of life for patients who receive IFN-α is worsened by heavy side effects, it may be necessary to identify beforehand which patients could benefit from IFN-α therapy [27].

Sacchi *et al.* [28] combined pre-treatment prognostic factors with the early response profile to IFN-α therapy. Response to IFN-α therapy at 3, 6 and 12 months was a significant predictor of later major cytogenetic response and the simultaneous presence of pre-treatment splenomegaly (≥ 5cm below the costal margin) or thrombocytosis (≥ 700 x 10^9/L) added a significant independent predictor to response.

Another kind of approach is now under investigation. A pilot study has recently recognized an association between HLA phenotype and response to IFN-α [29]. Patients with HLA B27 phenotype seem to have the best response rate to IFN-α; moreover, HLA-B 35, -A3 and -A31 show a trend towards a higher response. Further studies on a much larger number of patients are necessary to determine if the influence of HLA phenotype on survival is independent of other clinical prognostic factors.

Standard Chemotherapy

Busulfan (BU) until the mid eighties, and more recently Hydroxyurea (HU) were the two cytotoxic agents most commonly used in the treatment of CML patients. These drugs can control the signs and symptoms of CML but they do not have any effect on the progression of the disease. All patients who underwent traditional cytotoxic therapy evolved into blastic phase and died after a median survival of 3 to 6 years with a survival advantage for patients treated with HU over BU [30]. Other chemotherapeutic agents such as melphalan and thioguanine have been used in chronic phase CML patients but none of these has an advantage over HU. To date IFN-α is considered the golden standard of therapy for CML patients not suitable for allogeneic SCT; IFN-α can induce not only hematological response but also cytogenetic response.

Response Criteria

Before analyzing and comparing the results of IFN therapy, it is useful to list the criteria used for defining the Hematological Response (HR) and the Cytogenetic Response. Table 3 and 4 present the criteria adopted by the MD Anderson Group and by the Italian Cooperative Study Group (ICSG) on CML.

There are few differences in the definition of HR: in the first the absence of immature cells is required for Complete Hematological Response

Table 2. Prognostic Factors in CML

Patient associated	Older age Black race
Disease associated	Clinical: Symptoms at diagnosis Significant weight loss Hepatomegaly Splenomegaly Poor performance status Blood and bone marrow count: Anemia Thrombocytosis, thrombocytopenia, megakaryocytopenia Increased blasts, or blasts + promyelocytes in blood or marrow Increased basophils in blood or marrow Collagen or reticulin fibrosis grade 3-4
Treatment associated	Longer time to achieve hematologic remission with conventional chemotherapy High total dose of hydroxyurea therapy required in the first year to control disease Poor hematologic or cytogenetic response to IFN-α therapy

Table 3. MD Anderson's Criteria for Response to INF-α in CML

Response	Category	Criteria
Hematological response	Complete	Normalization of WBC count to < 9 x 10^9/L with normal differential Normalization of platelet count to < 450 x 10^9/L Disappearance of all signs and symptoms of disease
	Partial	Normalization of WBC with persistent immature peripheral cells, or splenomegaly or thrombocytosis at < 50% pretreatment level
Cytogenetic response	Complete*	No evidence of Ph+ cells
	Partial*	1% to 34% of metaphases Ph+
	Minor	35% to 90% of metaphases Ph+
	None	All analyzable cells Ph+

* Major cytogenetic response includes complete and partial cytogenetic responses.

(CHR), while in the second a < 5% percentage of immature cells is admitted.

With regard to cytogenetic response criteria, the differences are very small and the definition of cytogenetic response is quite similar in the two groups. The most significant result in CML studies is to obtain Ph+ clone suppression under 35%. The MD Anderson Group in fact denominates major cytogenetic response the sum of complete and partial cytogenetic response (Ph+ cells less than 35%) and the ICSG denominates complete and major cytogenetic response, achieving less than 35% Ph+ cells. Regarding less complete Ph+ clone suppression, the MD Anderson Group recognizes only minor cytogenetic response (35-90% Ph+ cells) while the ICSG distinguishes minor cytogenetic response (35-66%) and a minimal cytogenetic response (99-67%).

When single studies reported in this review utilize criteria significantly different from those presented in Tables 3 and 4, this will be specified.

Table 4. ICSG'S Criteria for Response to IFN-α in CML

Response	Category	Criteria
Hematological response	Complete	WBC < 10 x 10^3/μl and < 5% immature cells (including myelocytes, promyelocytes and blast cells) in the differential count Platelets < 450 x 10^3/μl Spleen not palpable
	Partial	WBC 10-19 x 10^3/μl, or 5-10% immature cells (including myelocytes, promyelocytes and blast cells) in the differential count, or Platelets 450-999 x 10^3 /μl, or spleen palpable (less than 10 cm below the costal margin)
Cytogenetic response	Complete	100% of Ph-negative metaphases
	Major	66-99% of Ph-negative metaphases
	Minor	34-65% of Ph-negative metaphases
	Minimal	1-33% of Ph-negative metaphases
	None	0% of Ph-negative metaphases

IFN-α Therapy

In 1983 Talpaz *et al.* initially reported that IFN-α was able to induce a cytogenetic response without causing bone marrow ablation [31].

After the first observations, IFN-α was extended to a larger number of patients. The results of the complete experience of the MD Anderson group were updated in 1995 [32]. A total of 274 patients with CML in early chronic phase were treated between 1982 and 1990 with $5MU/m^2/day$ of IFN-α or with the maximally tolerated dose. Of these, 219 (80%) achieved a CHR, with a cytogenetic remission in 159 of these (58%) (complete 26%, partial 12%, minor 20%). The estimated median survival was 89 months and the overall 5 years survival was 63%. A statistically longer survival was observed in patients achieving a cytogenetic response: the 5-year survival rates, dated from 12 months into therapy, were 90% for complete cytogenetic response, 88% for partial cytogenetic response, 76% for minor cytogenetic response. The MD Anderson experiences did not include a control group, treated with conventional chemotherapy. Nevertheless the survival advantage for IFN treated patients was remarkable in comparison with patients treated with conventional chemotherapy. Despite the significant differences in the pre-treatment performance status and degrees of the disease, only cytogenetic response at 12 months was associated with a better survival rate even if the probability of obtaining a complete or major response was higher for low risk patients, following Sokal's model [33].

Many studies from other groups confirmed the efficacy of IFN in CML.

Alimena *et al.* showed a CHR in 46% of 65 patients with a major cytogenetic response of 12% [34]. This study also demonstrated that a higher dose schedule of IFN-α had a statistically better rate of HR.

Another study was performed by the Cancer and Leukemia Group B (CALGB) with IFN-α at $5MU/m^2/day$ [35]. The overall median survival was 66 months, with a 5 year survival rate of 52%; no positive association of cytogenetic response with survival was found, but the number of patients with major cytogenetic response was low.

Mahon *et al.* recently reported the results of IFN-α treatment in 116 newly diagnosed CML patients [36]. The dose administered was $5MU/m^2/day$ and then adjusted to maintain the white blood cell (WBC) and platelet (PLT) count between 1.5 and 5 x $10^9/L$ and between 50 and 100 x $10^9/L$, respectively. A CHR was obtained in 80% of patients; 44% of patients had a major cytogenetic response; the estimated 5 year survival rate was 68%. The most significant factors associated with survival were the major and complete cytogenetic responses. These were independent prognostic factors compared with the Sokal score and baseline variables used to calculate the Sokal score. The achievement of a CHR and a minimal cytogenetic response within three months of therapy was a significant predictive factor for obtaining thereafter a major cytogenetic response.

Randomized Studies

Some randomized trials recently compared IFN-α with conventional chemotherapy. Three of these [37,38,39] clearly showed that IFN-α prolonged survival when compared with HU or BU. In all trials IFN-α achieved a higher percentage of major or complete cytogenetic response than the conventional chemotherapy did.

The Italian Cooperative Study Group randomized 322 patients to receive IFN-α at $5MU/m^2/day$ or HU/BU therapy [37]. Patients under 70 years of age with CML in early chronic phase were randomized on a 2:1 ratio of IFN to conventional therapy between July 1986 and July 1988. IFN-α therapy achieved a significantly higher incidence of major cytogenetic response (19% vs. 1%); the complete cytogenetic response was 8%. IFN-α was discontinued in 31% of patients, 16% for IFN-related adverse effects; 18% of the patients had a reduction of IFN dose higher than 50%. The time to progression from the chronic phase of leukemia to an accelerated or a blastic phase was longer in the interferon group than in the conventional-chemotherapy group (median, > 72 vs. 45 months; $P< 0.001$), as was survival (median 72 vs. 52 months; 6 year survival, 50% vs. 29%; $p=0.002$ for both comparisons).

Recently the results of a 10 year follow up of these patients have been published [40]. In the IFN arm 56 patients were alive (30%) versus 17 in the chemotherapy arm (18%). Forty-one patients were still receiving IFN-α; 9 of them were in complete cytogenetic remission and 11 in major or minor cytogenetic remission. Median and 10 year survival of low risk patients were 104 months and 47% in the IFN-α arm versus 64 months and 30% in the chemotherapy arm ($p < 0.03$). Median and 10 year survival of non low risk patients were 69 months and 16% in the IFN arm versus 46 months and 5% in the chemotherapy arm ($p < 0.006$).

At low Sokal's risk, hematological response and cytogenetic remission were associated with a longer survival. IFN treatment prolonged over 5 years did not show any unknown side effect and maintained a significant advantage in comparison with conventional chemotherapy. The authors concluded that a policy of chronic treatment with IFN-α maintained a significant survival advantage over conventional chemotherapy on a long-term basis and irrespective of the risk. However, the great majority of the long-term survivors were in the low-risk group.

The German/Swiss CML, study randomized 513 patients to receive IFN-α, HU or BU [41]. IFN-α and HU achieved better survivals than BU: the median survival was 66, 56 and 45 months, respectively ($p<0.01$); no difference was evident between IFN-α and HU; the median IFN dose was 3MU/m^2/day and IFN-α was broken off in 25% of the patients.

Among IFN-α patients only 7% had a complete cytogenetic response. In this study IFN was used in a low dose and 20% of the IFN patients were early discontinued without a sufficient long IFN therapy; besides, the study was performed on more advanced patients with symptomatic disease.

In the Medical Research Council (MRC) study all patients underwent a cytoreductive induction phase with HU, BU or BU plus thioguanine [39]. Then 587 patients monitoring a low normal WBC count for at least 4 weeks were randomized to receive IFN-α or HU/BU. Fifty-nine of 293 patients randomized to IFN-α had some cytogenetic response (complete 5%, partial 6%). The median survival times were 61 months for the IFN arm and 41 months for HU/BU arm ($p=0.0009$). Five year survival rates were 52% in the IFN arm and 34% in the chemotherapy arm. The 5 year survival rates were 100% in patients with complete cytogenetic response, 92% in patients with major cytogenetic response, 59% in patients with minor cytogenetic response and 47% in non responders. The median daily dose of IFN actually used was about 2MU/m^2.

A multicenter Japanese study compared IFN-α at the dose of 5MU/m^2/day with BU [38]. A total of 159 patients with CML in early chronic phase were randomized. A major cytogenetic response was obtained in 16% of the 80 patients receiving IFN-α versus 5% of the 79 patients receiving BU ($p=0.046$). The 5 year survival rate was 54% in the IFN arm and 32% in the BU arm ($p=0.02$). The survival was significantly longer in patients who had any cytogenetic response with either IFN-α or BU. Recently the same group published long-term follow up results, showing that the continuation of IFN-α may offer a survival advantage: a median survival of 71 months in the IFN-α group versus 55 months in the BU group ($p=0.05$) was observed. [42].

IFN-α was shown either in single arm studies or in randomized studies to be an effective therapy for early chronic phase (CP)-CML. Patients who had a complete or partial cytogenetic response showed better survival than patients who did not. Some studies demonstrated that the patients on IFN therapy who did not achieve a cytogenetic response had a survival equal to patients receiving a conventional chemotherapy [37,38,41]. In the MRC study patients treated with IFN-α survived longer than those treated with conventional therapy even if they had not demonstrably achieved a cytogenetic response [39]. Furthermore, the different studies showed a great variability in the rates of CHR and cytogenetic response. This is probably due to some differences in the distribution of different risk groups in these studies, e.g., the inclusion of advanced or accelerated phase patients, the number of cytogenetic studies and above all, the actual dose of IFN delivered. However, when patients were analyzed for IFN response and survival within each risk group, there were always a better results for IFN. Probably, the actual dose of IFN delivered is the main explaining factor and a strict relationship exists between IFN-α dose intensity and hematological and cytogenetic response rates [33].

Molecular studies, however, have found residual leukemic cells in almost all patients with IFN-α induced complete cytogenetic remissions [43]. Longer treatment may be necessary to maintain a stable PCR-negativity for bcr/abl transcripts, as recently observed after long-term cytogenetic remission (median of 42 months in 10 patients) [44]. On the other hand, it is to be noted that very low bcr/abl transcript levels have been detected in a significant number of normal adult blood donors [45].

In summary, controlled studies show that IFN-α increases life expectancy in patients who achieved major cytogenetic response, while patients who did not achieve Ph+ clone suppression had survival times equivalent to patients receiving conventional chemotherapy. Only CMR study showed a survival benefit with IFN also for patients not achieving cytogenetic response.

IFN in Combination with Hydroxyurea and Cytosine Arabinoside

In an attempt to avoid IFN resistance and increase the percentage of hematological and cytogenetic response, IFN-α has been used in combination therapy. Drugs most often used in association with IFN-α are HU and Cytosine Arabinoside (Ara-C).

A Benelux study utilized IFN-α at low doses (3MU, 5 days/week) associated with HU, compared randomly with HU alone in 195 patients [46]. The CHR at 6 months in the IFN group was 62% versus 38% in the control group. Cytogenetic responses in the IFN group were: 9% complete cytogenetic responses, 7% partial cytogenetic responses and 25% minor. No complete cytogenetic responses were seen in the group treated with HU alone; in this group 2 patients (2%) obtained a partial response and 8 patients (9%) a minimal response. In the IFN group both CHR and quality of cytogenetic response within the first 24 months were highly predictive for survival. In the IFN group HR and cytogenetic response were higher than in the control group but the duration of chronic phase from start of the study was not statistically different (53 vs. 44 months). Also no difference in a long term survival benefit was seen in two arms, probably attributable to too low an IFN dose. Thus HU doesn't improve cytogenetic response rate, nevertheless it is used to cut down WBC count before the start of IFN therapy.

Better results in combination therapy were achieved with IFN and Ara-C at low dose. The rationale of the Ara-C use is the proof of in vitro studies that Ara-C suppresses the growth of CML cells over that of normal hematopoietic cells [47].

Arthur *et al.* used IFN-α (5MU/m^2/day) and Ara-C (20 mg/m^2/day for 21 days repeated every 42 days) as first line treatment in 30 patients in early CP-CML [48]. All patients in the induction phase received HU with IFN-α. Cycles of Ara-C were repeated for 12 months, then IFN-α was continued alone. Patients achieved a CHR rate of 93% and a cytogenetic response rate of 67% (major 53%, complete 33%).

Positive data were achieved by CALGB study with IFN/Ara-C regimen (IFN-α 5MU/m^2/day and Ara-C 15 mg/m^2/twice a day as required) [49]. The initial dose of Ara-C was reduced to 10 mg/m^2 because of gastrointestinal toxicity. The results were: 72% CHR and 51% major cytogenetic response (15% complete cytogenetic response).

The Austrian group reported their data on an IFN-α/Ara-C regimen (IFN-α 3.5MU plus Ara-C 10 mg/m^2 for 10 days of a 28 day cycle) [50]. All patients received HU in the induction phase before maintenance therapy with Ara-C and IFN. Eighty-four patients were evaluable: there were 54% CHR, 25% cytogenetic response and 18% complete cytogenetic response. Overall survival at 12, 24, 36 and 48 months was 96%, 85%, 74% and 65%, respectively.

The French Chronic Myeloid Leukaemia Study Group conducted a clinical trial to assess the efficacy of combination therapy of IFN (5MU/m^2/day) plus monthly courses of Ara-C (20 mg/m^2/day for 10 days) [51]. All patients received an initial induction treatment with HU(50 mg/kg) and IFN. From January 1991 to May 1996, 721 patients were studied: 360 had been randomly assigned to the IFN-cytarabine group and 361 to the IFN group. The result was a significant benefit with combination therapy IFN plus Ara-C: the rate of CHR was higher in the IFN-Ara-C group: 66% versus 55% ($p=0.003$). A major cytogenetic response (complete + partial) was observed 12 months after randomization in 41% of patients treated with IFN and Ara-C and in 24% of patients treated with IFN alone ($p<0,001$). In the IFN group 118 patients who did not have CHR after 6 months, crossed over to receive Ara-C: cytogenetic responses were recorded in 18 patients (7 complete, 11 partial). In the IFN-α plus Ara-C group the patients who had a partial or complete cytogenetic response survived longer than the patients with no response or minor response ($p<0.001$); the same results were obtained in the IFN group ($p<0.001$). Furthermore longer survival was observed in the IFN plus Ara-C group. The 3 year survival rate was 85.7% in the IFN plus Ara-C arm and 79.1% in the arm of IFN alone. This study shows that the combination therapy in the early phase of CML achieves a superior cytogenetic response and survival than IFN-α alone. The problem with this combination is the compliance with daily subcutaneous injections. New oral formulation of Ara-C could probably solve the problem [52].

The MD Anderson Group treated 140 patients with early CP-CML with subcutaneous injections of IFN-α (5MU/m^2/day) and Ara-C (10 mg/day), compared with patients receiving IFN alone or IFN with intermittent Ara-C (7 days a month) [53]. The results showed a higher CHR with IFN plus daily

Ara-C than with IFN plus intermittent Ara-C or with IFN alone (92% vs. 84% vs. 80%, p=0.01). Furthermore the incidences of cytogenetic response were better with IFN plus daily Ara-C (74% vs. 73% vs. 58%, p=0.003) as were major cytogenetic responses (50% vs. 38% vs. 30%, p=0.06). However with a median follow-up of 42 months, the 4 year estimated survival rate for IFN plus daily Ara-C group was 70%, similar to those achieved with IFN-α with or without low dose Ara-C. This is in contrast to the results of the French CML Study Group [51] who achieved a significantly longer survival with the addition of low dosage Ara-C. The differences in these studies are probably due to different population characteristics, number of patients enrolled, follow-up time and the design of treatment schedules. The MD Anderson study showed a better survival rate in patients treated with IFN-α alone than the same patient group in the French Study (83% vs. 79% as 3 year survival rate). The French Study randomized a larger number of patients and therefore would have been more reliable in the estimation of outcome and detection of relatively small differences in survival.

Late Chronic Phase CML

Outcome with IFN-α therapies is well defined in early CP-CML, while little is known about the efficacy of IFN-α in late CP-CML, defined by a period of time from diagnosis to therapy longer than 12 months. The early experience with IFN-α therapy showed a decrease in responsiveness with increasing time from diagnosis [54].

Long term follow up results of 137 Ph+ patients in late CP-CML treated with different IFN-α based regimens has been recently reported [54]. IFN-α (5MU/m^2/day) was given alone to 22 patients or in combination therapy to the remaining patients as follows: IFN-α plus IFN-γ to 29 patients; IFN-α plus HU to 19; IFN-α plus intermittent low dose Ara-C to 67. Of the 123 evaluable patients 57% achieved CHR; 7% Partial HR (PHR) and 36% showed a resistant disease. Nineteen patients had a cytogenetic response: major (three complete and seven partial) in 10 patients (8%); minor in nine patients (7%). Eight out of the 10 patients who had a major cytogenetic response, were treated with IFN-α/Ara-C.

The median overall survival from start of therapy was 49 months, with an estimated 5 year survival rate of 41%. CHR and cytogenetic response rates were higher in the group of 67 patients (IFN plus Ara-C) than the other groups (IFN alone or IFN plus HU or IFN-γ). Despite an early trend for better survival with IFN-α plus Ara-C, the overall survival difference was not significant. The results of this study confirm that IFN-α has modest activity in late CP-CML.

A relationship may exist between CML progression and reduced efficacy of IFN-α. In vitro studies have shown that IFN-α increased the expression of cell surface adhesion molecules, which in cells from CML patients are decreased. These molecules prevent proliferation of Ph positive hematopoietic progenitor. It is possible that CML progression is associated with decreased cytoadhesion, increased proliferation and poor maturation [55].

The results of this long term follow up study [54] confirm that in late CP-CML the association of IFN-α and Ara-C is more effective than IFN-α alone, as previously shown by Kantarjian and Ferrajoli. Kantarjian [56] reported data regarding 60 patients with advanced CML phases (40 in late chronic phase and 20 in accelerated phase) treated with IFN-α (5MU/m^2/day) and intermittent low dose Ara-C. In these patients the results obtained were 55% CHR, 15% cytogenetic response and a 3 year survival rate of 75%. In this study the results obtained with combination therapy were better when compared to historical control group of patients treated with IFN-α alone.

Similar results were obtained by Ferrajoli *et al.* in a pilot study [57]; they treated 29 patients in late or accelerated phase with IFN-α (2-10 x 10^6 U/day) and intermittent low dose Ara-C. They also documented a suppression of secondary Ph+ clones which appeared during the previous treatment with IFN-α alone.

Management Approach for CML Patients

Based on all the previously reported data and on our personal experience, a flow chart for the management of early chronic phase CML patients is shown in Fig. (**1**).

Treatment of CML in Transformation

IFN has poor efficacy in patients with CML advanced phases. Nevertheless Kantarjian showed that IFN-α/Ara-C can induce a CHR in 50% of patients with clonal evolution as the sole diagnostic criterion for accelerated phase disease, with 20% of these patients achieving a cytogenetic response [56]. The most common approaches for patients in

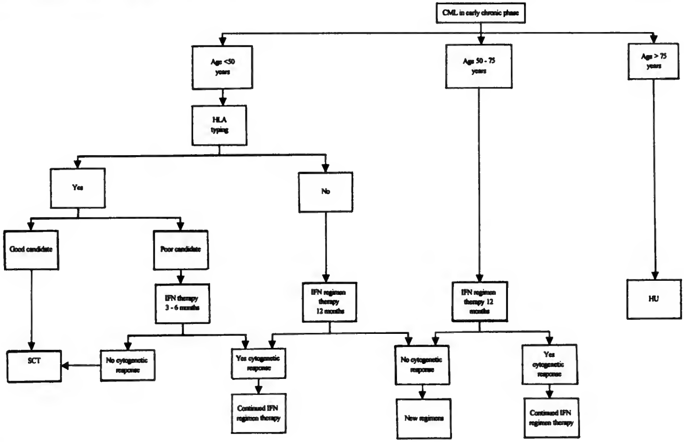

Fig. (1). Suggested management approach for CML patients in early chronic phase. The definition of a "good" or "poor" candidate is the result of a comples medicial decision making process based on: the Sokal and/or Synthesis prognostic score; risk assessment before allogenic SCT; personal feelings of the patient; personal experience of the physician caring for the patient.

blastic phase are intensive chemotherapy Acute Myeloid Leukemia-like, high dose Ara-C based [58], or, more recently, an alternative treatment with decitabine [59].

Other Treatment Approaches

Other drugs, alone or in combination have been used in CML and several showed some efficacy.

Homoharringtonine (HHT) is a synthetic derivative of a Chinese plant alkaloid, with potent myelosuppressive activity and little toxicity when used in a continuous infusion regimen. Initially used alone, subsequently HHT was utilized in 36 patients in late CP-CML resistant to IFN therapy, in combination with Ara-C. The dose schedule was: HHT 2.5 mg/m^2/day c.i. for 5 days and Ara-C 15 mg/m^2/day for 5 days sc. Using this schedule, 66% CHR, 34% cytogenetic response, 17% major cytogenetic response were obtained [60]. Based on these encouraging data, O'Brien *et al.* started a study on 90 patients with early CP-CML using HHT (2.5 mg/m^2/day c.i for 14 days until CHR and then for 7 days every month for 5 more courses) followed by IFN-α (5MU/m^2/day s.c.) [61]. The median follow up was 53 months. With HHT alone they obtained 92% CHR, 68% cytogenetic responses (27% major) and with the sequential treatment 44% major cytogenetic responses and 23% complete cytogenetic responses. A simultaneous HHT+IFN-α combination regimen was initially tested in late CP-CML and later begun in early CP-CML (HHT 2.5 mg/m^2/day c.i for 5 days every month plus IFN-α 5MU/m^2/day sc). The CHR was 89% and the major cytogenetic response was 37%, complete 16% [62].

In 1996, Cortes *et al.* [63] added the granulocyte-macrophage colony stimulating factor (GM-CSF) to IFN-α and showed that the combination therapy improved the cytogenetic response from minor to major in 3 out of 10 patients with IFN sensitive CML. These responses have been maintained for 4-6 years.

In 1997, Handa *et al.* [64], first described the effects of 13-CIS retinoic acid and IFN-α in vivo in 43 patients with CML. They obtained significant reduction of the WBC count and of the percentage of S-phase cells regardless of the stage of the leukemia. They also observed an increase in marrow apoptosis in two thirds of the patients and a suppression of bcl-2 and myc expression in a few cases.

Plicamycin has been used in association with IFN-α in a small number of patients with accelerated phase of CML. It appears to add efficacy to IFN in the stabilization of accelerated CML phase [65].

A pilot study of all-trans retinoic acid (ATRA) was performed by Cortes *et al.* [66] on 13 patients with CML in various stages, who had failed IFN-α therapy. ATRA was given orally at 175 mg/m^2 in two divided doses daily. A transient decrease in marrow and/or peripheral blood blast counts and promyelocites and/or basophyl percentage was observed in 6 patients. ATRA was well tolerated but ineffective in advanced IFN-α refractory patients.

An Italian group treated 18 CML patients in chronic phase with ATRA at a dose of 80 mg/m^2/day p.o., for 7 consecutive days every other week for a maximum of 12 course (1 course = 1 week on and 1 week off) [67]. Eleven patients went off the study because of progressive disease (8 patients) or refusal (3 patients). Seven patients completed the first 6 schedules of treatment with ATRA and two of them maintained a normal WBC count as induced by the treatment with HU; one patient completed the 12th schedule of ATRA in CHR. Alone ATRA appeared to be unable to control the WBC expansion in CML patients in chronic phase.

Most promising results have been obtained in vitro with the association of ATRA and IFN-α: this combination effectively inhibits the CFU-GM colony formations in peripheral blood progenitors from patients with CML [68,69].

Recently, 43 patients with early CP-CML were treated with a combination of IFN-α 5MU/m^2 plus low dose of Ara-C (10 mg /day s.c.) plus ATRA (45 mg/m^2/day). Response rate was substantially similar to those obtained with IFN-α plus Ara-C, but excessive toxicity was reported [70].

Finally, a pilot study on oral idarubicine was carried out on 14 CML patients in late chronic (13) or accelerated phase (1). CHR was achieved in 5 patients while no cytogenetic remission was obtained; the median survival was 44 weeks [71].

Others more experimental approaches, as bcr-abl-targeted therapies (bcr-abl antisense oligodeoxynucleotides, ribozymes, tyrosine kinase inhibitors) and CML-targeted therapies (RAS antisense, farnesyltransferase inhibitors, myb antisense oligodeoxynucleotides) are currently being investigated with encouraging early results.

Pegylated IFN-α

Polyethylene glycol (PEG) is a linear, hydrophobic, uncharged, flexible polymer which is available in a variety of molecular weights. PEG-modification of proteins represents a significant advance in protein pharmaceuticals. PEG-modification almost invariably lengthens the plasma half-life, reduces antigenicity and immunogenicity, and reduces sensitivity to proteolytic enzymes. The objective to produce PEG-IFNs is to increase the plasma half-life, and thereby to provide longer activity of PEG-IFN in comparison with IFN. The results of a phase I study of a PEG-IFN formulation in patients with CP-CML were presented at the 1998 ASH meeting in Miami [72]. Cohorts of patients received escalating doses of PEG-IFN-α ranging from 0.75 μg/Kg to 7.5 μg/Kg. Hematological remissions were achieved in 10 out of the 21 evaluable patients and further 8 patients showed an improvement in hematological response or achieved a partial response. The study included 13 patients who were previously resistant to IFN-α; 4 of these patients responded to PEG-IFN-α. Similarly, 2 patients who were intolerant of IFN-α were tolerant of PEG-IFN-α. These preliminary results suggest that PEG-IFN-α may be an appropriate alternative to IFN-α and in a near future it could substitute IFN-α.

Stem Cell Transplantation

Allo SCT is an effective curative option for patients with CML [73]. The International Bone Marrow Donor Registry and European Group for Bone Marrow Transplantation have recently [74,75,76] reported survival rates of 50-60% among patients with CML in chronic phase transplanted with an HLA-matched sibling donor. However this treatment is available to only 15% to 25% of patients because of age limitations and donor availability. Even if this kind of SCT is curative, it is associated with significant risk of early SCT-related mortality, from $< 20\%$ to $> 70\%$, and morbidity. Recently risk factors for CML patients before allogeneic SCT have been assessed. A simple system is now available based on donor type, age of recipient at transplantation, stage of disease, male recipient-female donor pairs and length of time from diagnosis to transplantation [77]. Utilizing this system and Sokal or synthesis model is now easier for physician caring for CML patients making adequate decision.

Results of SCT from a matched unrelated donor (MUD) are somewhat worse [78] but are also improving because of better matching strategies and supportive care and in selected patients, results are comparable with related donor outcomes [79]. Autologous stem cell transplantation is an appealing procedure and some good results have been recently published [80]. However this treatment still remains an experimental option.

Several studies have evaluated the effect of pretransplant IFN-α on related and unrelated donor transplant outcomes [81]. While one study with MUD has shown the IFN treatment for more than 6 months adversely affects survival, most studies with related donor transplantation have shown little or no adverse effects on outcome [81], in particular Hochhaus reported that IFN-α does not adversely affect the outcome of allogeneic SCT when stopped 90 days prior to the procedure [82]. Finally, in accelerated phase long-term survival after SCT is only 20-25% and survival after transplant performed in blast crisis further declines to 0% to 20% [81].

ESSENTIAL THROMBOCYTEMIA

Essential Thrombocytemia is a clonal process of the myeloid stem cell characterized by peripheral thrombocytosis.

The most generally accepted diagnostic criteria for ET are those described by Polycythaemia Vera Study Group (PVSG) in the mid 1970's, modified in 1997 [83,84]. The criteria required are listed in Table 5.

However more recently several authors trying to introduce positive criteria for ET diagnosis or to avoid the exclusion of early phases of this disease, proposed modifications of these criteria. The most important suggested features are: abnormal spontaneous megakaryocyte colony formation in the cultures of bone marrow, histological pattern of bone marrow with large megakaryocytes (with mature cytoplasm and multilobulated nuclei) and the tendency of such megakaryocytes to cluster in small groups close to sinuses. Recently, the value of platelet count has been shown not to be an absolute diagnostic criterion for ET [85,86,87].

The incidence of new ET cases is approximately 2.5/100,000 for year [88]. The median age is about 60 years. There is a female prevalence, with a 1.76:1 female/male ratio [89]. Genetic transmission is unusual, although some families with ET have been described [90].

In the clinical course, ET patients may be asymptomatic for long periods of time [91]. Otherwise two-thirds of ET patients are symptomatic presenting with vasomotor disturbances such as erythromelalgia, headache and dizziness. Furthermore thromboembolic manifestations with neurological symptoms such as strokes, TIA's, visual disturbances, seizures or hemorragic events such as hematomas and ecchymoses can frequently occur [92,93].

Even if there is no possible correlation between development of thrombotic or bleeding complications and laboratory findings [94,95], prior history of thrombotic events, advanced age and duration of thrombocytosis have been established as risk factors for subsequent thrombotic episodes [96]. Major bleeding is rare and seems to be related to higher platelet counts: a platelet count over 1500 X 10^9/L is generally considered an indication for cytoreduction.

Symptomatic patients are always treated, but it is extremely difficult to decide whether to treat asymptomatic cases. There is no evidence that asymptomatic patients, with only high platelet

Table 5. Diagnostic criteria for Essential Thrombocythaemia (PVSG)

- Platelet count greater than 600,000 μ/L
- No cause for reactive thrombocytosis
- Ht less than 40% or normal red cell mass (less than 36mL/Kg in males and less than 32mL/Kg in females)
- Normal red cell mean corpuscular volume or serum ferritin or marrow iron stain
- Collagen fibrosis of marrow absent or less than one third of biopsy area without both splenomegaly and leukoerytroblastic reaction
- No myelodysplastic changes on bone marrow smear
- No Ph chromosome or bcr/abl gene rearrangement

count, can benefit from myelosuppressive treatment [97]. These patients should remain under careful observation. Otherwise the therapy in asymptomatic patients with a history of previous thrombotic episodes and with cardiovascular risk factors and in symptomatic patients may prevent future complications and improve the symptoms [98,99].

Progression of ET to other chronic myeloproliferative syndromes, in particular to PV, has been observed in 5% of patients [97] and to acute myeloid leukemia has been observed in 3% to 4% of cases [85,94,100]. The terminal acute transformation is a part of the natural history of the disease but may be increased by treatment with radiophosphorus or alkylating agents, such as Busulfan. These drugs have been more or less abandoned.

Antiaggregating drugs, such as aspirin, have been shown to be effective in improving erythromelalgia, neurologic and visual symptoms when platelet count is high [92]. Reports of severe gastrointestinal bleeding have been attributed to aspirin in high dose in CMD patients with thrombocytosis [100,101,102].

Hydroxyurea, a non alkylating agent, is now considered the preferred treatment for ET patients. Continuous treatment with HU has been shown to reduce the platelet count, but in some cases it can cause granulocytopenia if the dosage is not appropriate. In the short term HU is considered safe and effective but some authors have reported that long time treatment with HU may induce secondary leukemia [103-106].

Therefore it is necessary to use other drugs, particularly in younger and low risk patients.

Anagrelide is the newest drug. It is the only drug approved by the Food and Drug Administration for clinical use specifically for the treatment of ET. It is an orally-administered, non mutagenic drug.

The Anagrelide Study Group has reported that this drug can reduce platelet count by at least 50% at a dose of 0.5 to 1.0 mg, four times/day in more than 90% of patients with thrombocytosis associated with myeloproliferative disorders [107]. However Anagrelide does not appear to be able to modify the myeloproliferative process, to reduce megakaryocyte mass, bone marrow fibrosis, splenomegaly. It is not well demonstrated that Anagrelide is able to reduce ET-related clinical symptoms and thrombotic risks. Some authors report that Anagrelide acts to inhibit megakaryocytic maturation [108]. Anagrelide can cause side effects, mainly related to vasodilatatory action including headaches, fluid retention and dizziness. The cardiac side effects include palpitations, tachycardia, arrhytmias and congestive heart failure. Less common side effects include diarrhea and abdominal pain.

The efficacy of IFN therapy in ET patients was first demonstrated by Giles in 1988 [109] and thereafter was confirmed by several authors (Table

Table 6. INF-α in Short Term Therapy of Essential Thrombocythaemia

Author (Ref.)	INF	Dose MU	Schedule	Cases	Hematological responses (%)
Lazzarino [111]	α-2b	1-4	Day	26	88
Gugliotta (reviewed in ref. [110])	α-2a	1-3	Day	27	100
Giles [114]	α-2a or α-2b	3-5	Day	22	100
Gisslinger [112]	α-2c	6-45	Week	20	85
Sacchi [110]	α-2b	3	Day	35	89
Other authors (reviewed in ref. [110, 111])	α-2a/2b/2c	3-9	Day	89	85
All patients reported				**219**	**91**

The criteria used by authors to evaluate the hematological response vary from study to study.

6). Sacchi *et al.* [110] treated 35 ET patients with IFN-α 3MU/day; after two months platelet count fell below 600 X 10^9/L in about 90% of the patients. Similar results were obtained by Lazzarino, Gisslinger, and Lengfelder [111,112,113]. The drug, non leukemogenic and non teratogenic, is able to reduce the platelet count, megakaryocytic mass and spleen size. Moreover it can control symptoms related to thrombocytosis. The most frequent IFN's used are recombinant IFN-α-2c, -α-2b and -α-2a. Dosage is different in induction phase and in maintenance phase; it varies from 1 to 10 MU three/seven times/week. The most frequent choice was 3MU three-seven times/week.

Even if the platelet count usually returns rapidly to the pre-treatment value after withdrawal IFN, in some patients long-term remissions are reported.

Giles [114] has documented sustained remissions without any therapy in two patients; Kasparu reported 3 patients in PHR after 3, 19 and 35 months after withdrawal of IFN-α [115]. In agreement with these results, Sacchi *et al.* reported three patients who obtained a sustained, unmaintained remission following long term IFN-α treatment [116]. In addition, Lengfelder's metanalysis showed that IFN-α dosage could be reduced during maintenance, but sustained, unmaintained complete remissions were achieved in only 12% of patients [113]. According to Sacchi *et al.* the only factor able to predict sustained remissions seems to be the clinical response to a low dose of IFN-α during the maintenance phase, rather than features before treatment [116]. Only 50% of patients who had platelet count below 450 X 10^9/L for at least 2 months with 3MU once a week obtained long-term remission when IFN-α was withdrawn.

Recently Gilbert [117] has shown the efficacy of IFN-α in a large group of patients with myeloproliferative disorders, treated with IFN-α 5MU/day in the induction phase, and 2.5MU/day in maintenance phase. The median duration of unmaintained response after the first course of IFN-α therapy was 60 weeks, but some patients were maintained successfully off treatment for ≥ 2 years.

In all relapsed patients, IFN-α was re-started and CHR was re-established with 3MU of IFN-α three times/week.

In summary, IFN-α was shown to have therapeutic activity in ET by several single-arm studies. However, to date no prospective randomized trials comparing IFN to conventional treatments (HU, anagrelide) have been performed. Available results showed the efficacy of IFN-α in maintaining a long term disease control with a low dose maintenance therapy. Sustained unmaintained remissions are reported after discontinuation of IFN-α in only a small number of patients.

A recently published study from Sacchi *et al.* assessed the magnitude of the effect of IFN-α on the neoplastic clone in ET [118]. Eleven female ET patients were treated with IFN-α at a dose of 3-6 MU/sc/day for 6 months. Ten of 11 patients obtained a CHR and one a PHR. The microspectroscopic analysis of ET megakaryocytes demonstrated a profound effect of IFN-α on the neoplastic clone even if the clonality studies always showed the persistence of clonal hematopoiesis. The failure to obtain a reconstitution of polyclonal hematopoiesis, which is demonstrated in some CML patients, can be attributed to IFN-α dose schedules. Restoration of a polyclonal methylation pattern was observed in CML patients after prolonged IFN-α treatment at a dose as high as 5MU/m^2/day. Whether a higher IFN-α dose and/or more prolonged IFN-α therapy may allow a restoration of polyclonal hematopoiesis in ET, remains to be determined.

To date, IFN-α cannot be considered as first line treatment for all ET patients. The side effects are often so severe as to require discontinuation of treatment in up to 25% of patients; the cost of IFN-α makes it an unlikely treatment for low-risk ET; in high-risk ET patients the risk/benefit analysis may favour HU over IFN-α for older patients and Anagrelide over IFN-α for younger patients. IFN-α may be considered a first choice in high-risk women of childbearing age because it is not leukemogenic or teratogenic and, of course, in patients who failed treatment with HU and Anagrelide [119,120].

Finally, a recently published study about monoclonal myelopoiesis in ET adds other subjects for discussion [121]. The results obtained in 23 patients (this study is possible only in women), suggest that ET, as diagnosed by the standard clinical and laboratory criteria, is a heterogeneous disorder in terms of clonality. No difference between patients with monoclonal and polyclonal myelopoiesis was recognized with respect to age at diagnosis, platelet count, incidence of hepato-splenomegaly or hemorrhagic complication. On the contrary, the incidence of thrombosis was significantly higher in patients with monoclonal disease. If this report is confirmed in larger studies, management of ET patients should be influenced by the study of clonality with a different approach to the treatment, based on clonality results.

POLYCYTHEMIA VERA

Polycythemia Vera is a clonal disorder of the hematopoietic stem cell with excessive proliferation of erythroid, myeloid and megakaryocytic cell lineages. It is characterized by absolute increase in red blood cell mass and total blood volume. In contrast to secondary erythrocytosis, in PV serum Erythropoietin (Epo) levels are usually low and erythroid proliferation is not Epo dependent [122,123]. The incidence of new cases is somewhat similar to that of ET. The median age at the time of diagnosis is 60 years; patients less than 25 years old are rarely reported [124]. There is a male prevalence with a 1.2:1 male/female ratio [125]. The importance of genetic factors in the origin of this disease is emphasized by several case reports of family history of PV [126].

The PVSG- required criteria for diagnosis are listed in Table 7 [85,124].

The causes of secondary polycythemia, like tumours and cardiac or pulmonary disease are to be ruled out before diagnosis. Specific attention must be paid to iron status because iron deficiency due to occult gastrointestinal blood loss may limit the increase in red cell mass and hide the diagnosis [127].

Frequently PV patients are asymptomatic; they can have splenomegaly, isolated erythrocytosis or thrombocytosis. In contrast to ET patients, untreated PV patients worsen; Chevitz reports that half of the patients die within 18 months of the start of symptoms if the hematocrit is not lowered [128].

Besides an asymptomatic phase, a hyperproliferative phase can be individuated, with erythrocytosis, thrombocytosis, leukocytosis. PV is by itself a slowly, progressive disease. About 10-20% of patients progress to a "spent phase", a stage characterized by marrow fibrosis with extra medullary hematopoiesis and pancytopenia [129]. About 2-15% of patients develop acute myeloid leukemia [130].

Thrombosis and hemorrhage are the most important clinical complications and major causes of morbidity and mortality [131,132]. In PV patients the frequency of venous and arterial thrombosis is similar. Risk factors for vascular complications in PV include previous history of vascular events and increased age [133,134].

A major cause of death is thrombosis, both cerebral and coronary [105]. Current treatments of PV have dramatically improved the survival of patients in the pretransformed stages. The median life expectancy in untreated PV patients varies from 6 to 18 months from diagnosis; on the other hand using citoreductive and antithrombotic drugs the median survival rises to over 10 years [133]. Therapeutic options include phlebotomy, phlebotomy in combination with myelosuppressive drugs, such as radiophosphorus (^{32}P) and alkylating agents, non alkylating agents such as HU and, more recently, IFN and Anagrelide.

It is important to underline that there are few randomized clinical trials on PV.

More than twenty years ago PVSG initiated a series of studies on the treatment of PV [135]. A total of 431 patients were randomized to one of the following treatments: 1) phlebotomy alone; 2) ^{32}P plus phlebotomy and 3) chlorambucil plus phlebotomy. Patients randomized to the first arm showed a higher incidence of thrombosis particularly in the first three years of the treatment. Subsequently the thrombotic rate in all three arms was found to be the same. In addition, phlebotomy

Table 7. Diagnostic criteria for Polycythaemia Vera (PVSG)

A
Red cell mass in mL/Kg greater than 36 in males and greater than 32 in females
Arterial oxygen saturation greater than 92%
Splenomegaly on palpation
PV diagnosis requires all three A criteria or two A criteria and any two of the following B parameters.
B
Platelet count greater than 400,000/μL
WBC count greater than 12,000/μL with no fever or infection
Raised neutrophil alkaline phosphatase score greater than 100
Raised unsaturated B12-binding capacity greater than 2,200 pg/mL

alone did not improve thrombocytosis, leukocytosis and splenomegaly.

On the other hand, after 3.5 years, therapy with ^{32}P and chlorambucil induced a significant increase in acute terminal leukemia and cancer of the gastro intestinal tract and the skin. For this reason, patients treated with phlebotomy alone had an overall median survival better than patients treated with chlorambucil and ^{32}P (12.6 years for phlebotomy, 9.1 years and 10.9 years for chlorambucil and ^{32}P).

Phlebotomies in association with 300 mg/day of aspirin and dipyridamole do not reduce the frequency of thrombotic complications in comparison with phlebotomy alone; on the contrary the risk of gastrointestinal tract bleeding increases [83,136].

HU, a non alkylating drug, in association with phlebotomy, is able to control thrombocytosis and high hematocrit of PV patients and can reduce the risk of thrombosis, as demonstrated also in ET by Cortelazzo *et al.* [137,138,139]. However, some authors have reported cases of acute leukemia following treatment with HU in PV patients [106,140]. Since 1984, Donovan has advanced questions about the mutagenic potential of HU [103]. Nand *et al.* analyzed 48 PV patients and showed that any myelosuppressive treatment, including HU, is associated with an increased incidence of acute leukemia [141]. Recently, an increase of acute leukemia in a long term prospective study of 50 patients (30 PV, 10 ET, 10 MMM) treated with HU has been demonstrated [106].

In 1997, the French Polycythemia Study Group published the results of maintenance therapy using HU after induction with ^{32}P in 461 patients greater than 65 years of age [142]. Low dose HU (5 to 10 mg/ Kg/ day) significantly prolonged the duration of ^{32}P-induced remissions but neither the rate of serious vascular complications nor the risk of progression to myelofibrosis were decreased; the leukemia rate was significantly increased beyond 8 years and also the rate of carcinomas was increased. Life expectancy was shorter (a median of 9.3 years vs. 10.9 years with ^{32}P alone); only cases with more rapid recurrence improved with the use of HU maintenance treatment, reducing the vascular risk and prolonging survival.

Kaplan did not agree with the idea that HU is a leukemogenic drug; he found that, after a follow-up of 378 weeks, HU was not associated with an increased risk of leukemic transformation [139], while the risk of thrombotic events was reduced.

Anagrelide has been used in thrombocytosis associated with PV, reducing megakaryocytosis and platelet count. However it has no effects on erytroid and myeloid cell lineages proliferation [143].

IFN-α seems to be an effective and safe treatment option for PV. The efficacy of IFN therapy in PV patients was first demonstrated by

Table 8. INF-α in the Treatment of Polycythaemia Vera

Author (Ref.)	INF	Dose MU	Schedule	Cases	Hematological responses (%)
Silver [148]	α-2b	3-m^2	3/Week	11	100
Cacciola [145]	α-2a	3	Day	11	82
Papineschi [147]	α-2a	3	3/Week	11	100
Turri [146]	α-2a	3	Day	11	64
Sacchi [143]	α-lymph.	3	Day	22	100
Foa [150]	α-2a	9	Week	38	50
Heis [151]	α-2c	12	Week	32	30
All patients reported				**136**	**75**

The criteria used by authors to evaluate the hematological response vary from study to study.

Silver in 1988 and thereafter was confirmed by several authors (Table 8) [144-149]. IFN-α is able to control myeloproliferation in PV patients, reducing platelet count and the requirements of phlebotomy in up to 70% of cases. Dosage varies between 3 and 5 MU three to seven times/week.

Silver, updating his results, reported on 11 PV patients treated with IFN-α 3MU/m^2 MU three times /week. In a follow up over 36 months they achieved good control of red cell values and elimination of the need for phlebotomy. No thrombohemorrhagic events have occurred, in contrast to a 40% incidence of significant thrombotic complications in the phlebotomy group during the first 3 years of therapy reported by the PVSG [148].

Sacchi *et al.* designed a trial comparing the ability of IFN-α plus phlebotomy and phlebotomy alone to control PV disease in 22 patients [149]. They chose a crossover study, in which patients received two treatments in sequence, as this method can produce statistically and clinically valid results with far fewer patients than would be required in a parallel study. In this trial, they treated PV patients alternately for 5 months each with phlebotomy plus 3MU/day sc of IFN-α and with phlebotomy alone. During IFN treatment, red blood cell count and hematocrit level were well controlled in both trial groups, reducing or eliminating the need for phlebotomy in all patients; furthermore, PLT number and WBC count declined during IFN-α therapy. In addition, the number of symptomatic patients was greatly reduced, and in six patients a reduction in splenic size was observed. The results demonstrated unequivocally that IFN is superior to phlebotomy in controlling myeloproliferative process and all the clinical aspects of the disease. In particular, IFN-α treatment at a dose of 3MU/day for 5 months produced a decreasing spleen size in 6 out of 14 patients (43%) and a reduction of abnormal proliferation of erythroid elements in bone marrow biopsies in 14 out of 21 patients (67%). All the patients symptomatic for pruritus (except one), acroparesthesias, acrocyanosis, peripheral vascular ischemia, obtained resolution of the symptoms at the end of IFN treatment.

Foa *et al.* reported long term therapeutic efficacy of IFN-α in 38 PV patients [150]. In responsive patients they obtained normalization of leukocyte count, platelet count and spleen size, a significant improvement of pruritus (10/10 patients). A complete response, defined as persistence of normal haematocrit without venesection was achieved in 11 patients (28.9%) and a partial response, defined as > 50% reduction of phlebotomy requirement, was achieved in 8 patients (21%).

Heis *et al.* [151] described the effect of IFN-α therapy on 32 PV patients. The dose of IFN-α administered was 12MU/week in the first year, 9MU/week in the second year and 12MU/week thereafter. They achieved normalization of platelet and leukocyte counts in a majority of patients, together with a reduction of phlebotomy frequency and incidence of deep venous thrombosis.

Furthermore, two reports [152,153] showed chromosome improvement in PV patients. The complete cytogenetic remissions obtained after IFN-α therapy suggest that this treatment can eradicate abnormal clone in PV patients.

These clinical findings are supported by in vitro studies showing reduction of circulating CFU-GM and BFU-E during the first two months of therapy in most patients [154]. In addition IFN-α inhibits megakaryopoiesis and antagonizes the action of platelet-derived growth factor (PDGF), a product of megakaryocytes which initiates fibroblast proliferation [155]. IFN-α might modify the natural history of PV and reduce or delay the development of secondary myelofibrosis in PV patients. To date, as for ET patients, IFN-α can not be considered as a usual treatment for PV patients. This drug could be used either in special subsets of patients or as an experimental treatment.

MYELOFIBROSIS WITH MYELOID METAPLASIA

Myelofibrosis with myeloid metaplasia is a chronic myeloproliferative disorder in which stem cell proliferation is associated with high degree of ineffective hematopoiesis. This leads to peripheral blood pictures ranging from refractory cytopenia to an excess number of circulating blood cells. A wide spectrum of bone marrow pictures has also been described, ranging from hypercellularity with an excess of megakaryocytes to depletion of all series of hematopoietic elements associated with large degrees of fibrosis. The major components of the fibrosis of the bone marrow (BMF) are collagen types III, IV and I [156]. The mechanism causing the formation of BMF is still unknown. The fibroblasts producing collagen in MMM are polyclonal [157,158]; they are stimulated to proliferate and to overproduce collagen by platelet derived growth factor (PDGF) and transforming growth factor beta liberated by megakaryocytes

[159,160]. Furthermore typical MMM features include immature granulocytes and erythroblasts in the peripheral blood, distorted and teardrop-shaped red blood cells, spleen and liver enlargement due to extramedullary hematopoiesis. The diagnostic criteria for MMM are still not universally accepted. We report in Table 9 the criteria formulated by the Italian Consensus Conference on Diagnostic Criteria for MMM in 1999 [161].

The average age at diagnosis of MMM is approximately 60 years. There is no evidence of sex predominance or genetic transmission.

Approximately 20% of patients are asymptomatic. The most common symptoms in MMM are related to anemia and spleen enlargement. Patients can suffer from weakness, fatigue, dyspnea, palpitations for the first and delayed gastric emptying or early satiety for the second. Bleeding complications can appear during the history of the disease. The course of disease is highly variable and median survival time from diagnosis ranges from 1.4 to 9.1 years [162]. Most patients die from infections, hemorrhages and chronic heart failure; sometimes MMM evolves into leukemic phase.

The disease is in practice incurable; there is not a specific and effective treatment. The treatment is based on the phase of the disease, mainly on supportive care with red cells and platelets transfusions. Other therapeutic approaches are directed to avoid or to diminish the number of transfusions administered.

Androgens stimulate erythropoiesis but the development of hepatic dysfunction or virilizing side effects may limit long term treatment.

Chemotherapeutic regimens (Busulfan, Thioguanine, Chlorambucil, Hydroxyurea) can be useful during hyperproliferative phases decreasing the risk of thrombosis or hemorrhages in the presence of thrombocytosis and sometimes reducing the spleen size.

Splenic irradiation has also been frequently used for treatment of pain caused by an enlarged spleen; in very selected cases, such as hemolysis and severe thrombocytopenia, splenectomy is indicated, even if there is an high operative risk, a liver enlargement is often to be expected and a high rate of blastic transformation is observed [163].

More recently, vitamin D3 has been used in MMM. It might inhibit formation of fibrosis and increase its degradation; nevertheless it can produce side effects, such as hypercalcemia. Also, growth factors, such as Epo and GM-CSF have been used in MMM patients [164]. The beneficial effect of Epo in MMM has been demonstrated by some authors, nevertheless the number of patients treated is very small [165,166]. Epo is useful in some MMM patients, especially those with inappropriately low levels of serum Epo. However some cases of aggravation of splenomegaly have been reported due to the stimulation of extramedullary erythropoiesis.

IFN-α has a large series of biological effects and some of these are theoretically useful in the treatment of MMM: inhibition of proliferation of the megakaryocytic cell line, inhibition of the activity of CSF-1 that is linked to the degree of myeloid metaplasia, regulation of the immune activation that is present in MMM, inhibition of types I and II collagen and fibronectin synthesis [167,168]. Thus the treatment with IFN-α in MMM would be

Table 9. Diagnostic Criteria for Myelofibrosis with Myeloid Metaplasia (Italian Consensus Conference, ref.n. 161)

A Necessary criteria
Diffuse bone marrow fibrosis
Absence of Philadelphia chromosome or bcr-abl rearrangement in peripheral blood cells
MMM diagnosis requires both A criteria plus any other two of B parameters if splenomegaly is present, or both A criteria plus any other four of B parameters when splenomegaly is absent
B Optional criteria
Splenomegaly of any grade
Anisopoikilocytosis with tear-drop erythrocytes
Presence of circulating immature myeloid cells
Presence of circulating erythroblasts
Presence of cluster of megakaryoblasts and anomalous megakaryocytes in bone marrow sections
Myeloid metaplasia

expected to cause inhibition of myelopoiesis, reduction of myeloid metaplasia and reduction of fibrosis in bone marrow [164].

However, in vivo results obtained in MMM with IFN-α therapy are poor and controversial. To date, approximately 70 MMM patients, in different stages of the disease treated with IFN-α have been reported in the literature (Table 10) [169-175].

A significant decrease of PLT count during IFN-α therapy was observed in patients with a hyperproliferation type of the disease, while WBC count rarely decreased; after withdrawal of the therapy a recurrence of thrombocytosis was always observed [174,176]. Hemoglobin (Hb) values increased in a small number of patients, but some of them showed a dramatic rise in Hb with a restoration of normal Hb level starting from severe anemia [177]. A decrease in spleen size was reported in less than half of the cases, above all in cases treated in the hyperproliferative state [176]. A reduction in bone marrow fibrosis has been reported in a few cases. Two reports about reversal of BMF have been published, in 1994 by Dalla *et al.* [178] and in 1998 by Domingues *et al.* [179]. The effective induction dose of IFN-α was 5MU/day and a smaller dose of the drug could be used for maintenance treatment for patients with MMM and thrombocytosis.

The limited results in MMM do not indicate IFN-α as a first-line therapy. IFN-α is an effective cytoreductive drug in the hyperproliferative phase of the disease and should be used only in younger MMM patients with the aim to avoid the leukaemogenic effects of other chemoterapeutic agents (HU, BU).

A new interesting approach was reported by Bourantas *et al.* in 1996 [180]. They used a combination therapy with Epo, IFN-α and GM-CSF in seven patients suffering from MMM. The dose-schedules were: Epo 200 U/Kg/3 times week, IFN-α 3MU/3 times week for 3 to 6 months, GM-CSF 250 $\mu g/m^2$/day for 3 weeks. All patients showed an increase of Hb levels and two of them were no longer dependent on red cell transfusions; a decrease of splenomegaly was observed in 4 patients and 2 patients had a decrease in BMF. This combination therapy is probably more effective when it is initiated soon after diagnosis.

Long-term multicentric studies are necessary to evaluate IFN-α and related-treatments because MMM is a rare disease.

PREGNANCY

Myeloproliferative disorders are described in pregnant women.

Antiproliferative drugs should be avoided because of their potentially adverse effects on the fetus; elective abortion is to be considered when an unexpected pregnancy is discovered during chemotherapy. According to its anti-angiogenesis

Table 10. INF-α in the Treatment of Myelofibrosis with Myeloid Metaplasia

Author (Ref.)	INF	Dose MU	Schedule	Cases	Hematological responses (%)
Parmeggiani [169]	α-2c	3	Day	2	0
Hasselbach [170]	α-2b	3.5	3/Week	10	0
Seewann [171]	α-2c	0.5-2	Day	5	20
Turri [172]	α-2a	3	Day	5	0
Gisslinger [173]	α-2c	25	Week	5	100
Barosi [174]	α-2b	3	Day	14	86
McCarthy [175]	α-2b	3	3/Week	8	0
Other authors ([177, 178 and other reviewed in [164]	α-2a-α-2b	0.5-3.8	3/Week to day	15	93
All patients reported				**64**	**50**

The criteria used by authors to evaluate the hematological response vary from study to study.

of patients achieved CHR and up to 70% a cytogenetic response. In a randomized trial Guilhot [51] reported survival benefit for patients treated with this combination.

IFN-α can be considered as an important treatment in several subsets of ET and PV patients. Sometimes IFN-α can be considered as first line therapy, for example during pregnancy, in high-risk women of childbearing age, in younger ET patients who failed treatment with HU and anagrelide, in younger PV patients in combination with phlebotomy to reduce the risk of leukemic transformation.

IFN-α may be associated with significant side effects that reduce enthusiasm for the use of this drug, produce difficulties in patient compliance, and, especially in CML, limit the dose delivered to the patient, thus resulting in an ineffective use of IFN-α. The rationale behind the development of new formulations of IFN-α (PEG-IFN-α), lies in more convenient administration and possibly an improved efficacy and toxicity profile. The active agent is slowly released following injection of PEG-IFN-α, thus resulting in constant serum levels of IFN-α over a prolonged period. The significantly longer half-life means that it can be administered once a week instead of once a day and hence it is much more convenient for the patient. The preliminary results of studies suggest that PEG-IFN-α may be an appropriate alternative, especially in patients who are resistant to, or intolerant of, standard IFN-α.

ABBREVIATIONS

Ara-C = Cytosine arabinoside

ATRA = All trans retinoic acid

BMF = Bone marrow fibrosis

BU = Busulfan

CHR = Complete hematological response

CMD = Chronic myeloproliferative disorders

CML = Chronic myeloid leukemia

CP-CML = Chronic phase of chronic myeloid leukemia

Epo = Erythropoietin

ET = Essential thrombocythemia

GM-CSF = Granulocyte macrophage colony stimulating factor

HHT = Homoarringtonine

HR = Hematological response

HU = Hydroxyurea

ICSG = Italian Cooperative Study Group

IFNs = Interferons

MMM = Myelofibrosis with myeloid metaplasia

MUD = Matched unrelated donor

PDGF = Platelet derived growth factor

PEG = Polyethylene glycol

Ph = Philadelphia

PHR = Partial hematological response

PLT = Platelet

PV = Polycythemia vera

PVSG = Polycythaemia Vera Study Group

SCT = Stem cell transplantation

WBC = White blood cell

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The Immune System and the Effects of Non-volatile Anesthetics on Neutrophil Transmigration Through Endothelial Cell Monolayers

Roland Hofbauer*[1,3], Alan D. Kaye[3], Stylianos Kapiotis[1], Oswald Wagner[1] and Michael Frass[2]

[1]Department of Medical and Chemical Laboratory Diagnostics, [2]Department of Internal Medicine I, Medical Intensive Care Unit, University of Vienna, Austria. [3]Department of Anesthesiology and Intensive Care Medicine, Texas Tech University Medical Center, Lubbock, TX.

Abstract: Inflammation represents the consequence of capillary dilation with accumulation of fluid and transmigration of leukocytes into the surrounding tissue. Leukocytes play a major role in the defense system of the body against invading microorganisms. This defense system has a non-specific branch consisting of granulocytes and macrophages and a specific branch of lymphocytes. Granulocytes release cytotoxic compounds from their intracellular granules into their local environment when encountering microorganisms. This random destruction happens rapidly, but it may also harm healthy tissue of the body. Leukocytes patrol the body by circulating through the blood and lymphatic system ensuring a continuous surveillance which is a prerequisite for an efficient defense. Upon tissue damage and inflammation, leukocytes are recruited from the blood to sites of injury, and this trafficking displays exquisite specificity.

In the late 1890's, Metchnikoff noted the power of certain blood cells to move towards microorganisms and ingest them. In fact, leukocytes adhere to the endothelium of the blood vessels, and subsequently leave the circulation by transmigration through the intercellular junctions of the endothelial cell monolayer. Transmigration is driven by chemoattractants, a process known as diapedesis. Reversible adherence of leukocytes to the endothelium, basement membranes, and other surfaces is an essential event in the establishment of inflammation, whose molecular basis is beginning to be understood. Inflammation may become chronic in many pathophysiologic processes and disease states.

In long-term mechanically ventilated critically ill patients, non-volatile anesthetics are needed over a prolonged time period. Perioperative infections are a major cause of morbidity and mortality in critically ill patients. Therefore, the influence of non-volatile anesthetics and opioid agents on the immune system is of high interest.

After presentation of the different effectors of the immune system and their fluxes through the body, the aim of this review is to propose a general model of leukocyte transmigration through endothelial cell monolayers. It emphasizes in which way different non-volatile anesthetic drugs may affect the non-specific branch of the immune system, i.e. the leukocyte transmigration through endothelial cell monolayers.

1. NEUTROPHIL TRANSMIGRATION THROUGH ENDOTHELIAL MONOLAYERS

Introduction and Background

Inflammation represents the consequence of capillary dilation with accumulation of fluid (edema) and the locomotion of leukocytes from the intravascular space into the tissue [1-5]. Neutrophil transmigration is a central component of many inflammatory diseases and correlates with clinical signs and symptoms. Leukocytes participate in the defense system of the body against invading microorganisms. The body defense system has a non-specific branch and a specific branch. Leukocytes, especially granulocytes and macrophages, belong to the non-specific immune system, whereas lymphocytes belong to the specific immune system [1-2]. Macrophages, the other class of defense cells from the non-specific immune

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*Address correspondence to this author at Department of Anesthesiology, Vascular Research, Texas Tech University, Lubbock, TX 79430, Tel. (806) 743-2981, FAX (806) 743-2984; Electronical mail to: roland.hofbauer@akh-wien.ac.at

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system, are able to consume and to destroy microorganisms by phagocytosis or, similar to granulocytes, by the secretion of cytotoxic compounds [6]. However, macrophages may also act more specifically by collaborating with lymphocytes. The lymphoid system is represented by two cell types, B-lymphocytes and T-lymphocytes. B-lymphocytes produce antibodies that bind to foreign organisms and facilitate their destruction by activating the complement system, which in term can perforate the membrane, or by "Opsonizing" the microorganisms [6]. T-lymphocytes act mainly by cell-to-cell contact. A subpopulation of T-cells, the so-called cytotoxic T-cells, recognizes and kills cells and microorganisms bearing foreign antigen [7]. The second subpopulation, helper cells, modulates the activity of other cells in the immune response. Granulocytes (neutrophils, eosinophils, and basophils) release cytotoxic compounds from their intracellular granules into their local environment when combating bacteria [6]. Granulocytes, especially polymorphonuclear neutrophils (PMN), comprise the first line of defense of the immune system against various insults and serve as a major component of the acute inflammatory response. All of these leukocytes circulate through the blood vessels and lymphatic system ensuring a continuous surveillance which is prerequisite for an efficient defense [1]. During tissue damage or inflammation, leukocytes are recruited from the blood to sites of injury [2-5]. PMN selectively enter sites of acute inflammation. A key component of the acute inflammatory response involves receptor-mediated migration of PMN out of the circulation and across a monolayer of endothelial cells in response to a variety of tissue-derived stimuli, e.g. bacterial toxins or tumor necrosis factor (TNF). The random distribution of PMN and other subsets of leukocytes happens rapidly, but it may also harm healthy tissue of the body. The cells of the immune system must both circulate as non-adherent cells in the blood and the lymph, and migrate as adherent cells into the tissue when necessary. Rapid transposition between adherent and non-adherent states is the key to the dual functions of the immune surveillance and responsiveness. An exception is the spleen, where small penicillar arterioles may terminate as open-ended vessels in the parenchyma, thus allowing a continuous unhampered access of blood leukocytes without need for adherent states.

Triggering of Leukocyte Adhesion

The inflammatory reactions which provoke the appearance of adhesion molecules [8] are mediated by proinflammatory cytokines. By the late 1940's, researchers detected such soluble molecules that arose at the site of bacterial infection. Some of them have also been found to modulate body temperature, e.g. interleukin 1 (IL-1) and interleukin 6 (IL-6) by acting on the brain. So called "alarm" cytokines are tumor necrosis factor (TNF-α), IL- 1 or interferon gamma (IFN-γ). Both IL-1 and TNF-α are mainly released by macrophages stimulated by microbial products, whereas IFN-γ is produced by T-cells. These different cytokines induce cell adhesion molecule expression on the vascular endothelium and on neutrophils. Another important family of cytokines are the chemokines. Chemokines are small chemotactic peptides with a molecular weight range of 8-10 kDa. These chemokines are produced by a variety of cell types [9]. They possess four cysteins conserved in all members of the family. α-chemokines are described as those where the first two cysteins are interrupted by one amino acid (C-X-C) and β-chemokines are described where the cysteins are together (C-C). The α-chemokines attract neutrophils, and β-chemokines act on eosinophils, basophils and monocytes. Further, both types of chemokines can react with a panel of transmembrane receptors on lymphocytes.

Adhesion Molecules

Adhesion of leukocytes to endothelial cells is regulated through a receptor-counterreceptor interaction, i.e. the adhesion molecules. Various types of adhesion molecule groups exist: selectins and their selectin ligands, integrins and their integrin ligands. Selectins are a family of transmembrane molecules, expressed on the surface of leukocytes and activated endothelial cells. Selectins contain a N-terminal extracellular domain with structural homology to lectins, glycoprotein substances, followed by a domain homologous to epidermal growth factor, and two to nine regulatory proteins (Table 1). Each of these adhesion receptors is inserted via a hydrophobic transmembrane domain and possesses a short cytoplasmic tail. During inflammation, the initial attachment of leukocytes from the blood stream occurs via the selectin family, and causes a small downstream movement of leukocytes along the endothelium, called leukocyte rolling. The selectin family includes L-selectin, the smallest of molecule family, and can be found on leukocytes. P-selectin, the largest selectin, is expressed on activated platelets and endothelial cells. E-selectin is expressed on activated endothelium with chemically or cytokine-induced inflammation. The smallest selectin, the L-

Table 1. Relevant Adhesion Molecules for Leukocyte Transmigration Through Endothelial Cell Monolayers

	Selectin	**Integrin**
Function	Initial cell-cell contact and cell rolling	Rolling and firm adhesion
Neutrophils	L-selectin (smallest)	β_1-integrin: VLA-4* β_2-integrin: CD-11a/CD-18* (= LFA-1) CD-11b/CD-18* (= MAC-1) CD-11d/CD-18* CD-11d/CD-18*
Endothelial Cells	P-selectin (largest) E-selectin (middle)	ICAM-1 (= CD-54) [binds to LFA-1 and MAC-1 on PMNL] VCAM-1 (= CD-106) [binds to VLA-4 on PMNL]

* most important one of the group
VLA-4: very late antigen
LFA-1: lymphocyte function-associate antigen
MAC-1: membrane adhesion complex
ICAM-1: intercellular adhesion molecule
VCAM-1: vascular cell adhesion molecule

selectin, is important for lymphocyte homing and adhesion to high endothelial cells of post capillary venules of peripherial lymph nodes. L-selectin is also known as LECAM-1 (lymphocyte-endothelial cell adhesion molecule) or LAM-1 (lymphocyte adhesion molecule). The largest selectin, the P-selectin, is expressed on activated platelets and endothelial cells. Other names for P-selectin include CD62P, GMP-140 (granule membrane protein), and PADGEM (platelet activation-dependent granule to external membrane protein). Within minutes of stimulation of the endothelial cells by inflammatory mediators such as histamine, thrombin, or phorbol esters, P-selectin is expressed on the surface. The selectin counterreceptors are PSGL-1 (P-selectin glycoprotein ligand) for P-selectin. PSGL-1 is a glycoprotein expressed on blood cells and contains sialyl-Lewis-X tetrasacceride. Another P-selectin ligand is CD24, which appears to be important for tumor cell binding to P-selectin. For L-selectin, four possible counterreceptors have been identified: GlyCAM-1 (glycosylation-dependent cell adhesion molecule), CD34, and MAdCAM-1 (mucosal addressin cell adhesion molecule), and also PSGL-1. The counterreceptor for E-selectin is not yet known. Integrins are a large family of heterodimeric transmembrane glycoproteins that attach cells to extracellular matrix proteins of the basement membrane or to ligands on other cells. Integrin contains large (α) and small (β) subunits. Some integrins mediate direct cell to cell recognition and interactions. Integrin contains binding sites for divalent cations (Mg^{2+} and Ca^{2+}), which are necessary for their adhesive function. Integrins are named in subgroups according to the subunits. β_2 -integrins are exclusively expressed on leukocytes and undergo a conformational change encompassing the phosphorylation of the β-subunit and contain four different heterodimers: CD-11a/CD18, the predominant β_2-integrin (also called LFA-1; lymphocyte function-associated antigen), CD11b/CD18 (also called Mac-1), CD11c/CD18 (also called p150,95), and CD11d/CD18. LFA-1 binds to the integrin counterreceptor ICAM-1 (intercellular adhesion molecule) on the endothelium. LFA-1 plays a crucial role in many cellular processes such as migration, antigen presentation, and cell proliferation. The most important subunit of the β_1-integrin subunits on leukocytes is VLA-4 (very late antigen). VLA-4 binds to the ligand and integrin counterreceptor VCAM-1 (vascular cell adhesion molecule). This interactions between VLA-4 and VCAM-1 are chiefly responsibly for adhesion to the vascular endothelium. VLA-4 is responsible for both leukocyte rolling and firm adhesion. For integrins, two counterreceptors exist, ICAM-1 (also called CD-54) and VCAM-1 (also called CD-106). These integrin counterreceptors, ICAM-1 and VCAM-1, belong to the immunoglobulin superfamily. ICAM-1 is constitutively expressed on resting endothelial

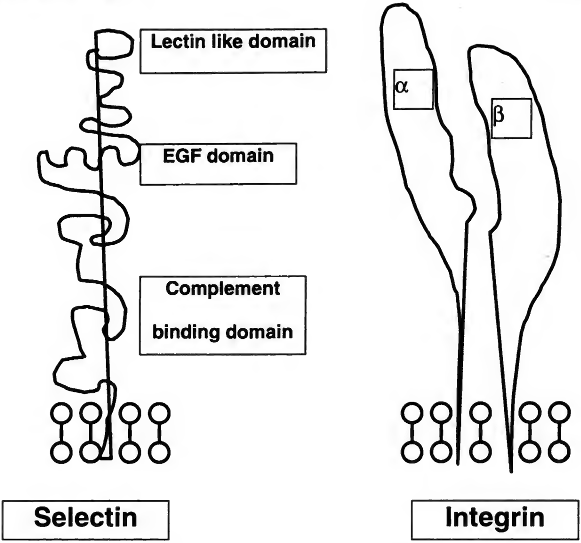

Fig. (1). Classes of adhesion molecules involved in leukocyte adhesion and transmigration.

cells, but is expressed extensively upon cytokine stimulation. ICAM-1 binds on the integrin LFA-1, and can also interact with Mac-1. ICAM-1 tends to arrest the rolling of leukocytes. VCAM-1 is a ligand for the integrin VLA-4. VCAM-1 promotes the adhesion of lymphocytes, monocytes, eosinophils, and basophils. VCAM-1 is a potent target for drugs [5-6, 8, 11-12] (Fig. **1**).

Transmigration Multi-step Model

In 1991, a multi-step model of leukocyte binding to the endothelium was defined by Butcher *et al.* [2]. Lawrence and Springer [13] showed also in 1991 that the first interactions between leukocytes and endothelial cell monolayers, the adhesion, were weak and neutrophils rolled gently along the vessel wall. This process is mediated by selectins. Selectins are a family of cell adhesion molecules consisting of a lectin-like domain, an epidermal growth factor-like domain, and variable numbers of domains that encode proteins homologous to complement-binding proteins. Selectins mediate the binding of leukocytes to the vascular endothelium [2, 8, 13]. When rolling neutrophils come in contact with chemokines, another family of adhesion molecules, the integrins, are expressed. These integrins are secreted by endothelial cells or other adjacent cells. Induced adhesion by activated integrins is tight and stops rolling. This important breakthrough leads to a four step model: 1) tethering (inflammation leads to vasodilatation and reduction in blood flow) and rolling of leukocytes; 2) triggering (turn on the smooth adhesion function to allow gently rolling of leukocytes); 3) strong adhesion (integrin induced strong attachment of neutrophils to the endothelium); and 4) transmigration (a rapid translocomotion of leukocytes through the intercellular junctions of the endothelial cell monolayers) [8, 14-18] (Fig. **2**).

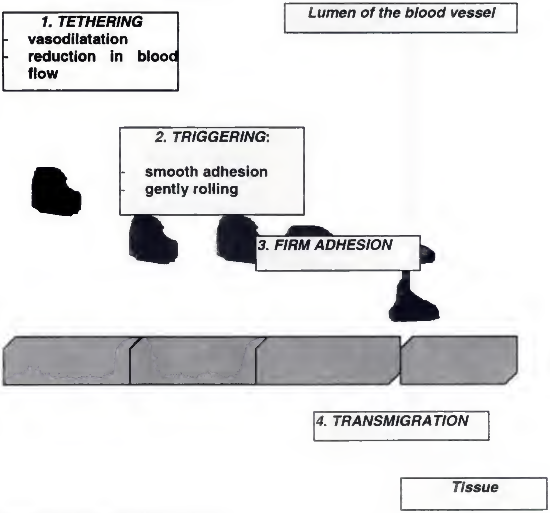

Fig. (2). Illustration of the transmigration process.

Leukocyte Recruitment

Leukocyte adherence to resting endothelial cells is poor. During inflammatory reactions, the phenotype of the endothelium is significantly modified. This change results from the contact with "alarm" cytokines, histamine or thrombin which induce the surface expression of molecules on the endothelium. First appearing are the P-selectins, continuously produced from endothelial cells and stored in the Weibel-Palade bodies. P-selectin allows step 1 of the multi-step model (tethering and rolling). After four to six hours of endothelial stimulation, E-selectin expression is mainly predominant in cutaneous inflammation [19]. After cytokine stimulation, endothelial cells produce chemokines and other factors (leukotriene LTB_4 and platelet activating factor, PAF) within minutes. All these substances are able to lead to integrin expression on leukocytes, which bind to the ICAM-1 (intercellular adhesion molecule) and VCAM-1 (vascular cell adhesion molecule) on endothelial cells, which are poorly expressed on resting endothelial cells. This integrin expression on leukocytes and endothelial cells needs only a few hours.

Inflammation and Transmigration

Many inflammatory diseases are characterized by migration of PMN into the endothelium. Examples of such pathophysiologic processes are commonly seen in the gastrointestinal and respiratory tracts, and the urinary and hepatobiliary systems [20].

Leukocyte Transmigration

Different scientific steps have led to sophisticated *in vitro* models. Endothelial cells are strongly involved in many important physiological, pathological, and physiopharmacological processes. However, up to the early 1970's, progress in endothelial cell research was very slow and could only be studied at a distance. Tissue culture as originally described by Harrison in 1907 was truly the "culture of tissues" and was performed by culturing bits of tissue in a plasma clot [21]. In 1910, Carrel and Burrows used Harrison's technique to culture cells of arteries from adult dogs and cats [22]. In the 1920's, Lewis observed the outgrowth of endothelial cells from cultured fragments of chicken embryo liver. By 1963, two independent research groups discovered the tissue explant methods to isolate endothelial cells from the vessels. Maryuyama perfused umbilical cords with enzymes and isolated endothelial cells that formed monolayers in cell culture. Unfortunately, cells did not replicate. In the early 1970's, the development of practical and routine methods for culturing endothelial cells radically altered this state of affairs and made possible a large number of experiments.

Development of Different Chemotaxis Models

Leukocyte locomotion can first be only a undirected activation of movement (chemokinesis), second, a directed movement along an increasing chemokine concentration (chemotaxis) [23], or third, a transmigration through a cell barrier, e.g. endothelial cell monolayer, along an increasing chemokine concentration (transmigration) [23]. The term "migration" is used in the literature either for chemotaxis or for transmigration. In this review, we use the term "transmigration" only when leukocyte locomotion through a monolayer of endothelial cells is studied.

Development of Comfortable Transmigration Assays

In the 1960's to the early 1990's, most investigators investigated leukocyte chemotaxis, i.e. leukocyte locomotion along an increasing chemokine concentration. Two different models were used during this time, an agarose assay and a three-dimensional cell chemotaxis model. In the agarose assay [24-26], leukocyte locomotion was investigated in a horizontal way. In contrast, three-dimensional cell chemotaxis models studied leukocyte locomotion in a vertical way from an upper compartment towards a lower compartment. One of these models used collagen gels [27-29]. Leukocytes were allowed to locomote from the upper compartment towards the gel placed below. Another three-dimensional cell chemotaxis model uses microporous filter systems [30-34]. In 1996, a comfortable three-dimensional leukocyte transmigration assay through endothelial cell monolayers was described by our group [35]. In 1998, a further development of this transmigration assay was used to investigate the influence of pharmacological drugs on leukocyte transmigartion [36-38]. In these studies, we have investigated the effects of many agents on leukocyte transmigration [36-38] through endothelial cell monolayers.

2. ANESTHETIC DRUGS AND THE IMMUNE SYSTEM

There are still many questions regarding the precise role of anesthetic agents with regard to infections and the immune system. Extended surgery, patient´s age, health status, and other factors may contribute to alteration of the immune system during anesthesia and surgery [39].

Reviewing the Reviews

There are very few reviews regarding the immune system and anesthetics in the literature. In the 1970´s, Lecky [40] for the first time described a possible influence of anesthesia and surgery to the immune system suggesting that bacterial growth and tumor spread may occur more readily, and host response to transplanted tissue and allergenic substances may be altered. In the early 1980´s, Cascorbi furthered these concepts [41]. In 1984, Lawman *et al.* [42] postulated that with increased use of chemicals in health related sciences, there is a need for a better understanding as to how these chemicals interact with the immune system. The authors reviewed the literature and collected information on the effects of chemicals on antibody-dependent cellular cytotoxicity. The major point emerging from this review was that there is conflicting literature and the mechanisms are not fully understood. In 1986, Simpson [43] published an interesting review of immune system and anesthesia. In 1987, Tomson [44] published a review on anesthesia and the immune system. This work discussed the effect of anesthetics on the immune system by evaluating patients undergoing anesthesia. In 1992, Kress *et al.* [45] published a review on effects of anesthesia and operation on essential immune functions. This group concluded that many findings, however, seem to point to an essential role of both the surgical trauma and the stress response in interactions with the immune system. By 1996, McBride *et al.* [46] published a review describing immunomodulation as an important concept in modern anesthesia. This review postulated that anesthetics might affect the immune system. McBridge came to the conclusion that more studies are necessary to understand the complex interactions.

Influence of Non-volatile Anesthetics on Cell Adhesion

In the early 1970´s, Giddon *et al.* [47] postulated that local anesthetics are able to suppress leukocyte adherence *in vivo*. In the mid-70's, Rabinovitch *et al.* investigated adherence of radiolabeled cells of a sarcoma cell line on adherence to glass cover slips under the influence of cationic anesthetics and tranquilizers [48]. This group found a decrease in cell adherence under the influence of anesthetics. One year later, the same group reported that cationic anesthetics are able to lead to a change in cell shape and speculated that anesthetics might influence the contractile cell machinery [49]. In 1977, Schiffer *et al.* [50] published an interesting study on the influence of local anesthetics to the adherence of granulocytes to nylon fibers. A dose-dependent reduction of granulocyte adherence to the artifical nylon fiber was demonstrated by this group.

Influence of Non-volatile Anesthetics on Leukocyte Adhesion

In 1987, a German group [51] published a paper on the influence of midazolam and ketamine on granulocyte adhesiveness using a nylon fiber assay, similarly to Schiffer´s investigation on local anesthetics [50]. In 1996, Miller *et al.* [52] investigated cytokine-induced neutrophil accumulation in rat mesenteric venules *in vivo* by general anesthetics. This group found a significant, dose-dependent reduction in neutrophil adhesion using thiopental, phenobarbital, ketamine, and some volatile anesthetics.

Influence of Non-volatile Anethetics on Leukocyte Functions

In the early 1970´s, different groups described the influence of local anesthetics on phagocytosis function of neutrophils [53]. Salo *et al.* was one of the first investigators who has systematically investigated the effect of thiopental on leukocyte phagocytosis function [54]. This work was followed by numerous investigations on leukocyte phagocytosis and respiratory burst from these agents [55]. All of these studies found that thiopental and ketamine inhibit leukocyte function. In the mid 1990's, Krumholz *et al.* [56] investigated the effect of different anesthetics, i.e. midazolam, droperidol, fentanyl, and alfentanil on leukocyte functions, e.g. phagocytosis and killing of bacteria *in vitro*, and found an inhibitory effect. In 1998, Heller *et al.* [55] published a work on elimination of Escherichia coli in human blood under the influence of the non-volatile anesthetics thiopental, propofol, midazolam, and ketamine. This group found that these anesthetics in particular at high concentrations inhibit the bacterial elimination. In 1998, Galley *et al.* [58] published the effect of midazolam and propofol on interleukin-8 (IL-8) release from neutrophils. A suppressive effect of the tested anesthetics on IL-8 release was observed. In 1998, Demiryurek *et al.* [59] investigated the effects of propofol on leukocyte production of reactive oxygen species and observed an inhibitory effect.

Influence of Non-volatile Anesthetics on the Movement of Leukocytes - Leukocyte Chemotaxis

The movement of leukocytes alone, tested in horizontal two-dimensional assays under the influence of non-volatile anesthetics was done more than twenty years ago. In 1976 and in 1977, Moudgil *et al.* published their findings on the influence of anesthetics on human leukocyte chemotaxis *in vitro* [60-61]. This group found a reducing effect in leukocyte migration, testing solely leukocyte movement. In the same year, Stanley *et al.* [62] published neutrophil chemotaxis during and after general anesthesia and surgery in 43 patients. This group found a reducing effect in leukocyte chemotaxis during anesthesia. Preoperative chemotaxis was also diminished by all anesthetics. The same reducing effect could be found postoperatively as well as preoperatively. However, Moudgil *et al.* described [63] the influence of anesthesia and surgery on neutrophil chemotaxis in patients requiring elective surgery using a three-dimensional cell chemotaxis assay in 1981. This group found a statistically significant reduction of leukocyte chemotaxis from an upper chamber into a lower chamber of the chemotaxis assay using thiopentone. This group studied leukocyte chemotaxis, i.e. endothelial cells were not included in the assay. In 1984, Moudgil *et al.* [64] demonstrated an inhibitory chemotactic effect secondary to volatile anesthetics. In 1985, Hammer *et al.* described [65] an inhibitory effect of local anesthetics on leukocyte mobility using an opto-electronic technique. In 1987, a German group showed [66] an inhibitory effect from intravenous anesthetics on neutrophil motility using a two-dimensional agarose assay. In 1992, Moudgil [67] published a comment on an article on anesthesia and leukocyte locomotion. For the past seven years, there has been little to no literature on this subject. Parallel to the investigations of our group on leukocyte transendothelial movement, a Japanese group [68] published a paper on an inhibitory effect of thiopental, midazolam, and ketamine on human neutrophil chemotaxis, without endothelial cell monolayers, and neutrophil functions in 1998. An inhibitory effect of these non-volatile anesthetics could be observed in this study.

Influence of Non-volatile Anesthetics on Endothelial Cell Functions

In 1989, Johns [69] described the effect of anesthetics on isolated rat thoracic aorta for the first time. This group found an inhibitory effect in vasodilatation using local anesthetics. In 1991, Shayevitz *et al.* [70] published the effect of anesthetics on isolated endothelial cells. This group studied the effect of volatile anesthetics on rat pulmonary artery endothelial cell viability. Various interesting papers about anesthetic effects on the endothelium followed in the next years. Research on anesthetic effects on the endothelium started at the begin of the 1990. In vivo, the endothelium in man has an intravascular and extravascular side. Only one study was done in respect to this subject by investigating anesthetic drug effects on endothelial cells. In the mid 90's, our group started to establish a cell culture research assay investigating anesthetic drug effects on leukocyte-endothelial cell interactions using a double chamber transmigration system. During the same time, Fischer *et al.* [71] used cultured endothelial cells in a double chamber cell culture system on a very-small pore microporous system and studied ion permeability of endothelial cell monolayers under anesthetics. Our group published a number of papers about the effect of various non-volatile anesthetics on leukocyte transmigration through endothelial cell monolayers *in vitro* [23, 72-73] in 1998. To our knowledge, there are no other studies investigating these transmigration phenomenon *in vivo*, neither in animals nor humans. The next sections of this review will focus on the effect of the non-volatile anesthetics ketamine, thiopental, and sufentanil on leukocyte transmigration through endothelial cell monolayers, *in vitro*.

3. INFLUENCE OF ANESTHETICS ON THE INTERACTIONS BETWEEN LEUKOCYTES AND THE ENDOTHELIUM: LEUKOCYTE TRANSMIGRATION THROUGH ENDOTHELIAL CELL MONOLAYERS

Ketamine

In long term ventilated patients in the intensive care unit, anesthetic drugs are needed over a longer time period. Ketamine is frequently used in critically ill mechanically ventilated patients [74]. Because of its cardiostimulatory properties, ketamine, a phencyclidine derivative, is used for anesthesia and sedation in sepsis or septic shock syndrome [75-77]. Little is known about the influcence of ketamine on leukocyte functions. Schmidt *et al.* [78] investigated the leukocyte adherence in mesenteric venules of rats anesthesized with ketamine. The endotoxin-induced leukocyte adherence was significantly reduced during ketamine treatment in postcapillary venules. We have investigated the effect of ketamine on leukocyte transmigration through endothelial cell monolayers [23]. In this study, an inhibitory effect of therapeutic plasma concentrations (3 µg/mL) on leukocyte transmigration through endothelial cell monolayers could be demonstrated.

Treatment of both cell types, leukocytes and endothelial cell monolayers, simulating the *in vivo* situation could lead to a decrease of leukocyte transmigration down to 52 ± 8.8 % compared to the bacterial toxin stimulated positive control ($p<0.05$). Formyl-methionyl-leucyl-phenylalanin (10^{-7} M) was used as bacterial toxin. The influence of different dosages (0.3, 3, and 30 µg/mL) led to a dose dependent effect. Fig. **3** shows the influence of ketamine in various concentrations.

To differentiate between undirected and directed migration, checkerboard analysis was done [23], and a directed migration of leukocytes could be observed using this *in vitro* transmigration assay.

This currently published study [23] supports the findings of Schmidt *et al.* [78] who described an inhibitory effect of leukocyte adherence in rat vessels. For leukocytes to enter the most extravascular sites of infections, they do not have only to adhere to endothelial cells, but also to transmigrate through a monolayer of endothelial cells. Ketamine significantly affects this transmigration, at least *in vitro*.

Several mechanisms might be responsible for the demonstrated inhibitory effect on leukocyte transmigration through endothelial cell monolayers. Pekoe *et al.* [79] described that ketamine impairs the leukocyte myeloperoxidase activity. Schmidt *et al.* [78] postulated an effect of ketamine on the myeloperoxidase reaction and an influence on TNF production. In addition to the TNF dependent adhesion molecule expression, a possible influence of ketamine on the expression of adhesion molecules on leukocytes or endothelial cells has been suggested by Schmidt *et al.* [79]. More studies are needed to focus this mechanisms in more detail.

Thiopental

It is well documented that thiopental affects leukocyte functions [80-82]. In these studies, the influence of thiopental on agarose gel leukocyte chemotaxis was investigated. A decrease of leukocytes chemotaxis to 38 % could be observed in these studies [80-81]. However, endothelial cell monolayers were not included.

Recently, we published the effect of thiopental on leukocyte transmigration through human endothelial cell monolayers in 1998. The model we used during this study was able to show a decrease in leukocyte transmigration through endothelial cell monolayers using a relevant plasma concentration of thiopental (10^{-5} M) down to 67 ± 9.8 % when both cell types, endothelial cells and leukocytes, were treated with thiopental ($p<0.05$ compared to control). Using different concentrations, a dose dependent effect could be observed (Fig. **3**). In our recently published study, the treatment of leukocytes alone did not lead to such a strong decrease of leukocyte transmigration as previous investigators could observe [80-81]. Our data suggest that the use of more complex *in vitro* models is helpful in describing and mimicking the complex *in vivo* situation [80]. Moreover, Nishida *et al.* [68] demonstrated that thiopental was able to reduce leukocyte chemotaxis in a microporous filter system in 1998. The method of Nishida *et al.* [68] avoided the artificial contact of leukocytes with agarose. This *in vitro* assay is one step closer to the *in vivo* situation, as the authors changed their assay from a two-dimensional to a three-dimensional system. In contrast, we used an *in vitro* assay for investigating leukocyte transmigration, including endothelial cell monolayers as partners of the migratory process [72].

To our knowledge, the influence of anesthetics on the immune system could only be studied in separate test systems, e.g. not simultaneously, until now. However, with each intravenous injection in patients, both cell systems, leukocytes and endothelial cell monolayers are in direct contact with thiopental. Hence, the investigation of thiopental effects may be regarded as a test allowing a physiological approach to study drug influence on both cell systems simultaneously. In this recently published paper [72] thiopental could be identified as a significant inhibitor on leukocyte transmigration through endothelial cell monolayers.

Sufentanil

The influence on leukocyte functions has not been investigated. Only little is known about the influence of sufentanil on cultured endothelial cell functions. In 1995, Fischer *et al.* [71] described a study on sufentanil and ion permeability of endothelial cell monolayers using a double chamber cell culture model. In 1998, we have published a paper on the influence of sufentanil on leukocyte transmigration through endothelial cell monolayers. The treatment of both cell types, endothelial cell monolayers and neutrophils, showed a reducing effect of approximately 20 % ($p<0.05$ compared to control) using a clinical relevant plasma concentration of sufentanil (5 ng/mL). Different concentrations of sufentanil (0.5, 5, and 50 ng/mL) showed a dose dependent effect (Fig. **3**).

Sufentanil could be identified as a potent inhibitor of leukocyte transmigration through endothelial cell monolayers. The opioid receptor antagonist naloxone may affect the influence of sufentanil on leukocyte transmigration. Manfreda *et al.* [83] published a paper on the chemotaxis of B-lymphocytes, as well as of T-lymphocytes. Although substance P-stimulated chemotaxis was not affected by naloxone, it prevented the chemotaxis of lymphocytes, thus confirming that the chemotaxis of lymphocytes is mediated by both opioid- and nonopiate-dependent mechanisms.

However, in this study of Manfreda *et al.* [83] only lymphocyte chemotaxis without endothelial cell monolayers was investigated. In the study on sufentanil and leukocyte transmigration [73], endothelial cell monolayers were included. We suggest that more complex *in vitro* models describe and mimic better the complex *in vivo* situation. In conclusion, sufentanil was identified as a potent suppressor of leukocyte transmigration through endothelial cell monolayers.

4. CONCLUSION

The influence of non-volatile anesthetics on leukocytes is well documented. Non-volatile anesthetics may inhibit leukocyte functions. Various functions, e.g. phagocytosis activity, respiratory burst, and interleukin release have been investigated well during the last 25 years, and are discussed controversially. More than 20 years ago, few investigators started to observe leukocyte movement under non-volatile anesthetics. During the last 20 years, these models developed from two-dimensional, more artificial systems, to three-dimensional double chamber assays.

The effect of non-volatile anesthetics on endothelial cell functions is poorly understood,

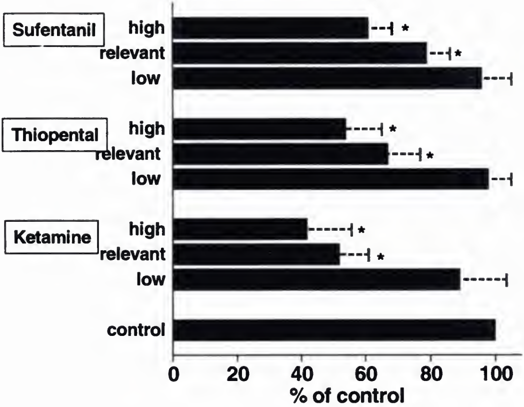

Fig. (3). Influence of ketamine, thiopental, and sufentanil on leukocyte transmigration through endothelial cell monolayers when both cell types, leukocytes and endothelial cells were treated, simulating a situation after an i.v.-application.

only limited data are available since about five years. The combination of leukocytes and endothelial cells and its simultaneous investigation under different drugs such as anesthetics is available since the publication on this subject in 1998.

Our investigations on leukocyte transmigration through endothelial cell monolayers could identify the non-volatile anesthetics ketamine, thiopental, and sufentanil as potent inhibitors on the fast immune branch of the immune system. Increasing insight into more complex *in vivo* situations is longing for more complex *in vitro* models to describe and mimic better the complex *in vivo* situation in scientific investigations. The future in this subject will be the development of intelligent cell co-culture systems.

Over the past few years, studies have demonstrated numerous results with regard to the mechanism of action of ketamine-induced responses in the lung. The development of arginine analogs which inhibit nitric oxide synthesis have facilitated the investigation of the role of nitric oxide in regulating vascular tone and responses in the pulmonary circulation. Nitric oxide synthesis inhibitors decrease pulmonary vasodilator responses to acetylcholine, enhance pressor responses to angiotensin II and hypoxia, and have a small effect on baseline pressures in the isolated perfused rat lung, suggesting that nitric oxide plays a role in mediating or modulating pulmonary vascular responses in the rat [84-87]. In one study in rat pulmonary arteries with chronic hypoxic pulmonary hypertension, ketamine had no effect on nitric oxide-mediated relaxation in either normal or pulmonary hypertensive rats [88]. Ketamine-induced responses in the rat and guinea pig via endothelium- and epithelium-independent pathways [89-90]. In the isolated rabbit portal vein, ketamine induced vasorelaxation was not endothelium dependent but rather was due to blockade of the voltage-gated influx of extracellular calcium [91].

Nitric oxide has been identified as the biologically active endothelium-derived relaxing factor (EDRF) [92]. Nitric oxide is synthesized by the vascular endothelium from the terminal guanidino nitrogen atom of the amino acid L-arginine and acts as a natural vasodilator [93]. The relaxation of arteries and veins is mediated by activation of guanylate cyclase and increasing cyclic guanosine 3',5'-monophosphate [94-95]. Nitric oxide is synthesized from L-arginine by platelets, cerebellar and peripheral neurons, neutrophils, and macrophages as well as by endothelial cells [96-97].

Although the exact mechanism by which ketamine dilates the pulmonary vascular bed is unknown, this phencyclidine derivative has significant pulmonary vasodilator activity. Ketamine produced dose-related decreases in the feline pulmonary vascular bed [89].

The exact mechanism by which ketamine-induces a vasodilator effect in the pulmonary vascular bed is not well understood, the results of a previous study suggested that this induction drug has significant pulmonary vasodilator activity at clinically relevant doses.

The effects of ketamine may involve an effect on calcium influx or release from the sarcoplasmic reticulum. In rabbit and guinea-pig preparations, ketamine inhibits L-type calcium channels, reduces calcium influx, and/or activates a calcium-ATPase [98-99]. However, in vascular smooth muscle cells, ketamine had no significant effect on calcium uptake into intracellular stores or on calcium extrusion [100]. Ketamine was also found to inhibit agonist-induced synthesis of inositol 1,4,5-triphosphate in rabbit mesenteric artery and inhibit the release of calcium [101]. A similar mechanism for the inhibition of calcium release was found in the rabbit femoral artery where ketamine decreased the release of calcium and reduced phospholipase C activity [102].

The exact mechanisms between possible nitric oxide, intracellular ion changes, and adhesion molecule regulation and expression resulted in the observed reduced transmigration of neutrophils through endothelial cell monolayers remains to be evaluated.

In conclusion, the immune response of patients receiving an operation is not attributable to surgery only. Many drugs are used during anesthesia have wide ranging immunomodulatory properties. There is evidence that non-volatile anesthetics seem to influence intracellular functions, e.g. intracellular ion changes or nitric oxide release. Recently, influence of non-volatile anesthetics on leukocyte transmigration through endothelial cell monolayers is well documented using high sophisticated cell culture *in vitro* models investigating cell-to-cell interactions. The achieved *in vitro* data over the last years might support the clinical observation that non-volatile anesthetic drugs influence the immune system.

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Adenosine and Ischemic Preconditioning

Bruce T. Liang*, Tomasz A. Swierkosz, Howard C. Herrmann, Stephen Kimmel, and Kenneth A. Jacobson[a]

Department of Medicine, Cardiovascular Division, University of Pennsylvania Medical Center, Philadelphia, PA

[a]Laboratory of Bioorganic Chemistry, National Institutes of Health, Bethesda, MD 20892

Abstract: Adenosine is released in large amounts during myocardial ischemia and is capable of exerting potent cardioprotective effects in the heart. Although these observations on adenosine have been known for a long time, how adenosine acts to achieve its anti-ischemic effect remains incompletely understood. However, recent advances on the chemistry and pharmacology of adenosine receptor ligands have provided important and novel information on the function of adenosine receptor subtypes in the cardiovascular system. The development of model systems for the cardiac actions of adenosine has yielded important insights into its mechanism of action and have begun to elucidate the sequence of signalling events from receptor activation to the actual exertion of its cardioprotective effect. The present review will focus on the adenosine receptors that mediate the potent anti-ischemic effect of adenosine, new ligands at the receptors, potential molecular signalling mechanisms downstream of the receptor, mediators for cardioprotection, and possible clinical applications in cardiovascular disorders.

I. ADENOSINE RECEPTORS

Four subtypes of adenosine receptors, A_1, A_{2A}, A_{2B}, and A_3 receptors, have been identified by molecular cloning [1, 2]. They all belong to the superfamily of G protein-coupled receptors and are characterized by seven hydrophobic membrane-spanning segments. The cloning and expression of recombinant adenosine receptors have been carried out in a number of species including chick, cow, dog, pig, rat, mouse, rabbit, sheep, as well as human [1]. In a given species, coding sequence of a particular adenosine receptor derived from different tissues is similar or identical. Thus, it is likely, although not proven, that an adenosine receptor subtype is the same whether it is isolated from the brain, heart or lungs.

The adenosine A_1 and A_3 receptors are G_i-coupled receptors; their activation leads to inhibition of adenylyl cyclase activity and of cyclic AMP accumulation [3]. Whether the A_1 or the A_3 receptor is also coupled to other G protein is not known. However, it is known that the A_1 receptor is also coupled to the atrial cardiac myocyte potassium channel (Kir) [4] and phospholipase C [5] although the $\beta\gamma$ subunit of G_i is likely the mediator linking the adenosine A_1 receptor and the Kir and phospholipase C. Recently, the A_3 receptor has also been shown to mediate phospholipase D activation [5]. The identity of the G protein in coupling the A_3 receptor to phospholipase D remains unknown.

The other two adenosine receptors, the adenosine A_{2A} and A_{2B} receptors, are coupled to G_s leading to stimulation of adenylyl cyclase activity and cyclic AMP accumulation [6]. However, the coupling of A_2 receptors is not limited to G_s. Thus, the A_{2B} receptor is also potently coupled to G_q leading to a pronounced stimulation of phospholipase C [7]. The physiological response mediated by each receptor-effector, for example, the A_1 receptor-phospholipase C, in a given tissue remains to be determined. The biological role of these specific receptor-effector interactions in the cardiovascular system remains an area of intense interest and deserves further investigation.

II. CHEMISTY AND PHARMACOLOGY OF ADENOSINE RECEPTOR LIGANDS

The structures and affinity of various agonists and antagonists are shown in Figs. **1** and **2**.

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*Address correspondence to this author at the 956 BRBII/III, University of Pennsylvania Medical Center, 421 Curie Blvd., Philadelphia, PA 19104, Tel: 215 573-2408, Fax: 215-662-2947, E-mail: liangb@mail.med.upenn.edu.

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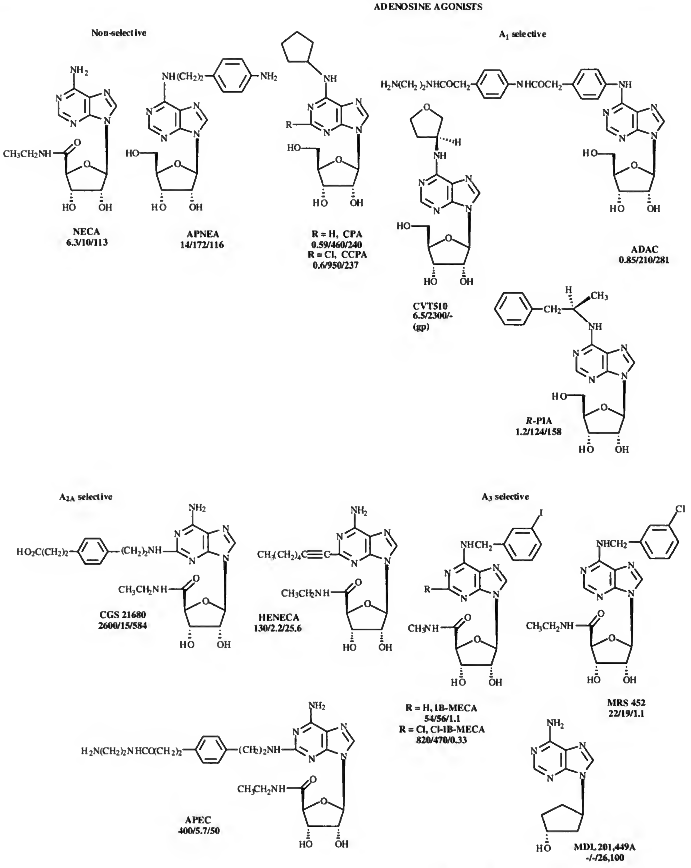


Fig. (1). Agonists at the various adenosine receptor subtypes. Non-selective as well as selective agonists are shown. K_i values, in nM, for A_1, A_{2A} and A_3 receptors (rat, unless indicated) are shown in that order below the structure and name for each compound.

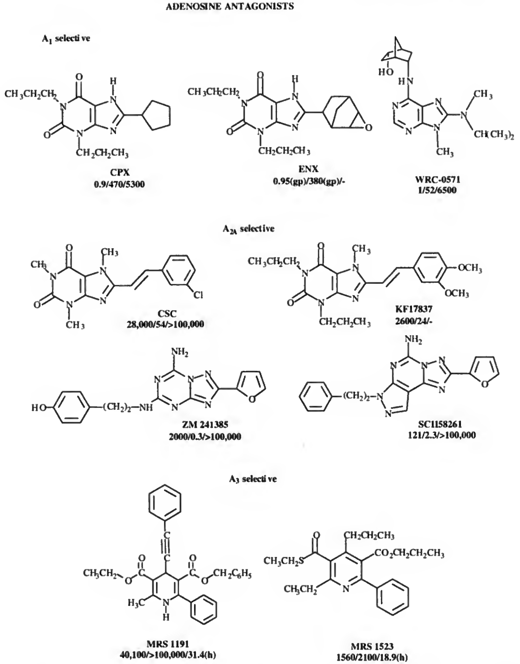


Fig. (2). Antagonists at the various adenosine receptor subtypes. The structure and K_i values for adenosine receptor antagonists are shown. K_i values, in nM, for A_1, A_{2A} and A_3 receptors (rat, unless indicated) are indicated in that order below the structure and name for each compound.

Non-selective Agonists

NECA (5'-N-ethylcarboxamidoadenosine) is among the most potent non-selective adenosine agonists and is still the most potent agonist reported at the A_{2B} adenosine receptor subtype, for which there are no selective agonists. Previously, NECA has been used erroneously as an A_{2A}-selective agonist [8]. Originally (1972) NECA was included in a patent filed by Abbott Laboratories for use in treating hypertension. As an adenosine analogue modified at the 5'-position of the ribose ring, NECA is not subject to phosphorylation by adenosine kinase.

APNEA (N^6-(*p*-aminophenylethyl) adenosine has been used for the activation of A_3 receptors [9], particularly in combination with A_1-receptor selective antagonists, however it is non-selective, and other agonists highly selective for the A_3 subtype are now available such IB-MECA (see below).

A_1 Receptor Agonists

Numerous adenosine derivatives substituted at the N^6-exocyclic amine with aryl, alkyl, or cycloalkyl groups have been found to selectively activate the A_1 adenosine receptor subtype. Such modifications preclude inactivation by adenosine deaminase and thus greatly prolong the half-life in pharmacological systems. Among the earliest potent adenosine derivatives was *R*-PIA ((R)-N^6-phenylisopropyladenosine), which is the chemical combination of adenosine and L-amphetamine (to form the phenylisopropyl group). In the late 1960's, prior to the discovery of its mechanism of action, *i.e.* adenosine receptors, this compound was reported to be a potential hypotensive agent. It was tested in humans, but the clinical development was curtailed due to side effects. While the hypotensive effect of this agonist is related to its activation of A_{2A} receptors, nevertheless, *R*-PIA is moderately selective for the A_1 subtype. In fact, in tritiated form it was introduced in 1980 as one of the first radioligands shown to bind specifically to A_1 adenosine receptors in the cerebral cortex [10]. *R*-PIA has been used as a selective A_1 agonist for studies of cardioprotection [11], however even more selective A_1 agonists are available and preferred, since in the heart the selectivity of A_1 adenosine agonists is generally diminished with respect to binding affinities at A_1/ A_{2A} receptors [12].

Among the more highly selective A_1 agonists are CPA (N^6-cyclopentyladenosine) and its 2-chloro analogue CCPA [13]. A homologous series of N^6-cycloalkyl adenosine derivatives has been shown to be highly potent and selective at the A_1 subtype [8], with optima occurring at cyclopentyl and cyclohexyl. Both CPA and CCPA have been used in various models of cardioprotection [14] and cerebroprotection [15]. Chronic administration of CPA in mice was found to mimic some of the CNS effects of acute administration of an A_1 selective antagonist [8]. CVT510 (N^6-((S)-3-tetrahydrofuranyl)adenosine) is an ether derivative structurally related to CPA, which is highly selective for the A_1 receptor and has been proposed for the treatment of reentrant tachycardias that involve the A-V node [16].

N^6-Phenyl substituted adenosine derivatives have been studied as receptor agonists. One such derivative, ADAC (adenosine amine congener, N^6-[4-[[[4-[[[(2-aminoethyl)amino]carbonyl]methyl] anilino]carbonyl]methyl]phenyl-adenosine), was introduced in the mid 1980's as a functionalized congener (i.e. it contains a primary amino group at the terminal position of a strategically placed elongated chain, thus allowing conjugation to carriers without losing receptor binding) with high affinity and selectivity for the A_1 receptor subtype [17]. ADAC has been used as a chemical precursor for spectroscopic, *e.g.* fluorescently-labeled, probes of the A_1 receptor. Also, its conjugation with bifunctional crosslinking reagents, such as *p*-phenylene diisothiocyanate (*p*-DITC), has resulted in irreversibly binding affinity labels for the A_1 receptor in the heart [18]. Tritiated ADAC was shown to be a high affinity radioligand for the A_1 receptor displaying minimal non-specific membrane binding, despite the presence of aromatic rings in the functionalized chain. ADAC is cardioprotective in a chick cardiac myocyte model of ischemia [19], and also cerebroprotective in a model of global ischemia [20]. Acute administration of ADAC at a very low dose range (25 - 100 µg/kg) was found to limit post-ischemic hippocampal damage in gerbils following bilateral carotid artery occlusion. Hemodynamic side effects of the adenosine agonist were minimal within this low dose range.

A_1 Receptor Antagonists

DPCPX (or CPX, 8-cyclopentyl-1,3-dipropylxanthine) was the first highly selective A_1 antagonist to be introduced [8]. Tritiated DPCPX has been used widely as a high affinity radioligand for the A_1 receptor. Although it is roughly 500-fold selective for the rat A_1 vs A_{2A} receptor, its use as an absolutely selective adenosine antagonist must be tempered by studies at the other two subtypes. The potency of DPCPX at A_{2B} receptors and at human (but not rat) A_3 receptors is intermediate (K_i values of approximately 30 and 75 nM, respectively) [21, 22]. It should be noted that, in general, the A_3 receptor affinity of most xanthines is highly species dependent, with the affinity at human receptors typically >100-fold greater than at rat receptors.

Curiously, DPCPX was found to have an unanticipated action in the promotion of chloride efflux in CFPAC cells, which contain a mutation of the CFTR chloride transporter (the F508) that occurs in cystic fibrosis [23]. DPCPX is in Phase II clinical trials by SciClone Pharmaceuticals for the treatment of cystic fibrosis, an action apparently not resultant from its A_1 receptor antagonist properties.

ENX (1,3-dipropyl-8-[2-(5,6-epoxy)norbornyl] xanthine) is a conformationally constrained structural refinement of CPX. This xanthine is highly potent and extremely selective for A_1 receptors in binding and functional assays [24]. ENX is a racemic mixture and was subsequently resolved. The pure S-enantiomer, CVT 124 (S-1,3-dipropyl-8[2-(5,6-epoxynorbornyl)]xanthine), which is the more potent isomer, is selective for A_1 receptors even with respect to the A_{2B} receptor. It is under development by Biogen Corp. for cardiac and renal applications.

In pharmacological studies, many synthetic xanthine derivatives have proven difficult to dissolve in biological media, for example, the aqueous solubility of DPCPX at neutral pH is 30 μM.

In the search for novel adenosine antagonists, a number of classes of non-xanthine heterocycles, which are amenable to structural optimization as subtype-selective antagonists, have been identified. Among non-xanthine antagonists which have been explored are numerous 9-alkyladenine derivatives, such as WRC-0571 (Martin *et al.*, 1996), developed by Discovery Therapeutics, which displays a high degree of selectivity for A_1 vs. A_3 receptors.

A_{2A} Receptor Agonists

Many A_{2A} agonists which are derivatized at the 2-position have been introduced. Perhaps the most widely used A_{2A}-receptor selective agonist is CGS 21680 (2-[4-[(2-carboxyethyl)phenyl]ethylamino]-5'-N-ethylcarboxamidoadenosine), which is modified at both 2- and 5'-positions (a NECA-like derivative). CGS 21680 has a K_i value of approximately 15 nM at both rat and human [26] A_{2A}-receptors and was introduced originally for the treatment of hypertension. It is 140- fold selective for A_{2A} vs A_1 receptors [27], and does not readily cross the blood-brain barrier, thus it is of great utility in cardiovascular studies. CGS 21680 is also highly selective for the A_{2A} vs A_{2B} receptor [28], although its affinity at A3 receptors is greater than its A_{2A} receptor affinity. Tritiated CGS 21680 is a useful high affinity agonist radioligand for the A_{2A} receptor displaying low non-specific membrane binding.

APEC is an ethylenediamine derivative of CGS 21680 which is even more potent at the A_{2A} subtype and is also moderately selective for this subtype. APEC is an amine-functionalized congener (see ADAC above), which does readily cross the blood-brain barrier [29]. Thus, administered peripherally it is suitable for the study of the function of A_{2A} receptors in the central nervous system. The covalent conjugate of APEC with *p*-aminophenylacetic acid (PAPA-APEC) is useful as a high affinity radioligand for A_{2A} receptors and was used for photoaffinity crosslinking in the first study to distinguish A_1 and A_{2A} receptors as distinct molecular species [30]. APEC has been used as a chemical precursor for a fluorescent probe of the A_{2A} receptor [31], and its conjugation with a bifunctional crosslinking reagent has resulted in an irreversibly acting A_{2A} agonist with coronary vasodilator properties.

HENECA (adenosine 2-(hexynyl)-5'-ethyluronamide), which is modified with an alkyne group at the 2-position as well as at the 5'-position, is a highly potent vasodilator and A_{2A} receptor agonist with a K_i of 2.2 nM in binding assays [32], although it is not as selective as CGS 21680 for the A_{2A} vs. the A_1 and A_3 receptors.

A_{2A} Receptor Antagonists

Among highly potent and selective non-xanthine antagonists for the A_{2A} subtype are ZM241385 (4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3*a*][1,3,5]triazinyl-amino]ethyl)-phenol) [33] and SCH 58261. SCH 58261 (5-amino-7-(2-phenylethyl)-2-(2-furyl)pyrazolo[4,3-e]-1,2,4-triazolo[1,5-c]pyrimidine) [34] is highly selective for A_{2A} vs. both A_1 and A_{2B} receptors and has been tritiated for use as a radioligand [35]. ZM241385 is the most potent of A_{2A} antagonists yet reported. Its selectivity vs A_{2B} receptors is not as high as the corresponding selectivity of SCH 58261. In fact, tritiated ZM241385, while a superb radioligand at A_{2A} receptors, is of sufficiently high affinity at recombinant A_{2B} receptors for use as a radioligand when these receptors are expressed in systems lacking A_{2A} receptors [21].

Although most 8-substituted alkylxanthine derivatives reported are selective for the A_1 subtype, 8-styrylxanthines have been demonstrated to be A_{2A} selective antagonists. KF 17837 (7-methyl-(8-(3,4-dimethyloxystyryl)-1,3-dipropyl-xanthine) is such a styrylxanthine having roughly 100-fold A_{2A} receptor selectivity that was introduced by the Kyowa Hakko Co [36]. KF 17837 and related A_{2A}-antagonists such as the 1,3-diethyl homologue, KW 6002 have entered clinical trials as potential novel treatments for Parkinson's disease. CSC (8-(3-chlorostyryl)caffeine) displays selectivity for A_{2A} vs A_1 and A_{2B} receptors of 520-fold and 62-fold, respectively [8].

A_3 Receptor Agonists

The first highly potent and selective A_3 agonists contain substitution at both 5'- and N^6-positions.

One of the most widely used A_3 receptor agonists is N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide (IB-MECA) which is 50-fold selective [37] for A_3 vs either A_1 or A_{2A} receptors *in vitro* and appears to be highly A_3 selective *in vivo* [38, 39].

The corresponding derivative which also contains a 4-amino group in the benzyl ring is I-AB-MECA, which is used widely as a high affinity radioligand for recombinant A_3 receptors [40], although in autoradiographic studies of rat brain sections it was found to label also A_1 and A_{2A} receptors.

The 3-chlorobenzyl derivative of NECA (MRS 452) has been demonstrated to be cardioprotective via the selective activation of A_3 receptors.

Substitution of adenosine analogues at the 2-position in combination with modifications at N^6 and 5'-positions was found to further enhance A_3 selectivity [41]. Cl-IB-MECA (2-chloro-N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide) displayed a K_i value of 0.33 nM at A_3 receptors and is selective for A_3 vs A_1 and A_{2A} receptors by 2500- and 1400-fold, respectively. A_3 agonists have been shown to both inhibit adenylyl cyclase and activate phosphatidylinositol-4,5-bisphosphate-specific phospholipase C, with a rank order of potency that reflects the order of affinity determined in binding assays but at generally higher concentrations than the K_i values in competition of binding [41, 42]. A_3 receptor activation has also been shown to be coupled to activation of phospholipase D [43]. There may be differences in the relative activation of various second messenger systems based on the concentration of the A_3 agonists. Low concentrations of A_3 agonists such as Cl-IB-MECA (<100 nM, Fig. **1**) have been shown to antagonize the A_1 receptor mediated inhibition of EPSPs in hippocampal slices [44]. High concentrations of A_3 agonists have been found to induce apoptosis [45], thus intense activation of the A_3 receptor may have a damage-inducing, rather than a protective role.

In human ADF cells of astroglial lineage, 100 nM Cl-IB-MECA caused a marked reorganization of the cytoskeleton, with appearance of stress fibers and numerous cell protrusions (which became enriched in the antiapoptotic protein Bcl-x_L), accompanied by induction of the expression of Rho, a small GTP-binding protein [46]. A high concentration of Cl-IB-MECA ($\geq$ 10 μM) was lethal to cultured rat cerebellar granule neurons, and the toxic effects of glutamate were also augmented.

A_3 receptors are present in relatively high density in human peripheral blood eosinophils. Cl-IB-MECA was found to be very potent in inhibition of eosinophil migration without affecting adhesion receptors CD18 and selectin or assembly of F-actin [47].

MDL 201,449A is a carbocyclic adenosine derivative that acts as a weak, selective A_3 agonist [48].

A_3 Receptor Antagonists

Since xanthines tended to bind only weakly to A_3 receptors, an alternate strategy, *i.e.* screening diverse molecules in chemical libraries for leads, was adopted in the design of selective A_3 receptor antagonists. The Merck group [49], using high throughput screening of heterocyclic compounds, has identified antagonists which are highly selective for human A_3 receptors.

1,4-Dihydropyridines, known as potent blockers of L-type calcium channels and used widely in treating coronary heart disease, were found to bind appreciably to human adenosine A_3 receptors [42]. Common dihydropyridine drugs typically bound either non-selectively (for example, nifedipine, with a K_i value of 8.3 μM) or in some cases with selectivity for the A_3 vs other adenosine receptor subtypes (for example, *S*-niguldipine, with a K_i value of 2.8 μM).

1,4-Dihydropyridines have been used as a template, in which it has been possible to select for affinity at adenosine receptors and completely deselect for affinity at L-type Ca^{2+}-channels. Such a compound is MRS 1191 (3-ethyl 5-benzyl 2-methyl-6-phenyl-4-phenylethynyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate), which has been found to inhibit radioligand binding at the human A_3 receptor with a K_i value of 31 nM, while the same derivative was nearly inactive in binding at A_1 and A_{2A} receptor sites (*i.e.* >1300-fold selective). MRS1191 is moderately selective at rat A_3 receptors, with a K_i value of 1.42 μM. It competitively antagonized the effects of N^6-(3-iodobenzyl)-5'-N-methylcarbamoyladenosine (IB-MECA,), an A_3 receptor selective agonist, on inhibition of adenylyl cyclase mediated by the recombinant human A_3 receptor. Dihydropyridine antagonists of A_3 receptors have also proven selective in chick cardiac myocytes, in which the activation of A_3 receptors induces protective anti-ischemic effects. MRS 1191 was also shown to be A_3 receptor-selective in the rat hippocampus, in which it was demonstrated that A_3 receptor activation suppresses the effects of activation of presynaptic A_1 receptors on inhibition of neurotransmitter release [44]. MRS 1191 was also utilized to demonstrate that presynaptic A_3 receptor activation antagonizes group III metabotropic glutamate autoreceptors [50].

Antagonism at human A_3 receptors by MRS 1191 was demonstrated in functional assays consisting of agonist-induced inhibition of adenylate cyclase and the stimulation of binding of $[^{35}S]GTP\text{-}\gamma\text{-}S$ to the associated G-proteins, with a K_B value of 92 nM [42].

A pyridine derivative, MRS 1523 (5-ethyl 2-ethyl-3-(ethylsulfanylcarbonyl)-4-propyl-6-phenylpyridine-5-carboxylate) is highly potent at human A_3 receptors [51]. It is one of the few A_3 antagonists reported to be selective and potent at both human and rat A_3 receptors, with K_i values of 18.9 and 113 nM, respectively.

MRS 1220 is an N^5-p-phenylacetyl derivative of the non-selective non-xanthine antagonist CGS 15943 (9-chloro-2-(2-furyl)[1,2,4]triazolo[1,5-*c*]quinazolin-5-amine). While MRS 1523 is an A_3 antagonist of broad application to various species, the triazoloquinazoline MRS 1220, is extremely potent in binding to the human (Ki 0.65 nM) but not the rat A_3 receptor [42].

Recently, a series of isoquinoline derivatives such as VUF8504 were reported to be antagonists selective for human A_3 receptors [52].

III. TYPES OF ANTI-ISCHEMIC CARDIOPROTECTION

Adenosine-mediated cardioprotection can be separated into two types. The first type of cardioprotection is an acute effect in which the presence of adenosine during an episode of ischemia can protect the cardiac myocyte against the deleterious effect of ischemia. The second type of adenosine-mediated cardioprotection is ischemic preconditioning in which adenosine can produce a protective effect with a "memory" that confers ischemia resistance on the cardiac myocyte long after adenosine or adenosine agonist has been removed.

A. Acute Cardioprotective Effect of Adenosine

In this type of cardioprotection, activation of adenosine receptor during acute ischemia can produce a potent anti-ischemic effect. The advantage of this type of cardioprotection is that activation of adenosine receptor in the ischemic cardiac myocyte can delay or prevent myocyte death and thus produce a beneficial effect even after ischemia has begun. Recent studies show that either A_1 or A_3 receptor can mediate such an acute cardioprotective effect [19]. It is not unexpected that the first identified and the most well-characterized adenosine receptor in the myocardium, the adenosine A_1 receptor, is capable of mediating such protection. However, the finding that activation of the adenosine A_3 receptor can exert an acute cardioprotective effect during ischemia is novel and suggests that the adenosine A_3 receptor is also an important mediator of the protective action of adenosine in the heart.

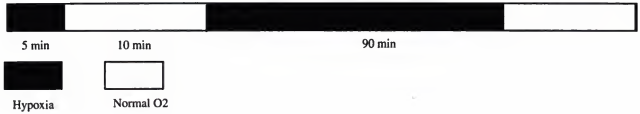
B. Adenosine and Ischemic Preconditioning

The original observations of ischaemic preconditioning were made in animals. As described before, Murry *et al.* [53] observed that repetitive short periods of ischaemia rendered the myocardium of dogs less susceptible to a subsequent prolonged and otherwise lethal episode of ischaemia. This seminal observation provoked studies proving that ischaemic preconditioning can limit infarct size in dogs [54-56], pigs [57], rabbits [58,59], and rats [60].

Evidence for such protective preconditioning also exists in humans (61-63). Adenosine plays a key role in triggering and mediating the cardioprotective effect of ischemic preconditioning in most animal species including humans [53, 64-66, 61, 54, 67, 62, 68-71, 63, 72]. Studies of adult human and rabbit ventricular myocytes and cultured neonatal rat and embryonic chick cardiac myocytes provided important insights by indicating that the cardioprotective mechanism of preconditioning is exerted, at least in part, at the level of and by the cardiac myocytes in the intact heart [73-75].

Using a cardiac myocyte model of simulated ischemia and ischemic preconditioning (Fig. **3**), Liang *et al* [3] provided the first direct evidence that the cardiac myocyte expresses functional adenosine A_3 receptors and that the A_3 receptor can mediate the protective effect of ischemic preconditioning. Thus, in addition to the A_1 receptor, the A_3 receptor is also capable of mediating preconditioning. These data raised the question of why cardiac myocytes have two adenosine receptor subtypes that appear to mediate similar cardioprotective functions. The most recent evidence suggests that, on the contrary, the adenosine A_3 receptor serves a different

(A) Ischemic / Hypoxic Preconditioning



(B) Pharmacologic Preconditioning

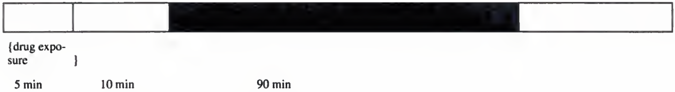


Fig. (3). Preconditioning Protocol. The protocol has been used in most of the experimental studies on ischemic preconditioning. The preconditioning studies carried out in patients undergoing PTCA also employed a similar protocol.

cardioprotective function as compared to the A_1 receptor [14]. While activation of both A_1 and A_3 receptors can mimic the protective effect of ischemic preconditioning, the A_3 receptor can mediate a more sustained cardioprotective effect, as manifested by a more prolonged duration of its preconditioning-mimicking effect.

IV. SIGNAL TRANSDUCTION MECHANISM LEADING FROM ADENOSINE RECEPTOR ACTIVATION TO CARDIOPROTECTION: A WORKING MODEL

To facilitate the determination of the signalling mechanism(s) underlying the cardioprotective effect of ischemic preconditioning, the process of preconditioning can be divided into two phases (Fig. **4**). The first phase is the trigger or initiation, which occurs during the first episode of ischemia that is brief in duration. The second phase is the actual exertion of protection during the subsequent, second episode of ischemia that is prolonged and that is responsible for the injury to the cardiac myocyte. The second phase is also known as the maintenance phase of ischemic preconditioning. The release of adenosine and the subsequent activation of adenosine receptors are responsible for both initiating and exerting the cardioprotective effect of ischemic preconditioning. Although much more work is needed, accumulating evidence is emerging to support a role of phospholipase C or D, protein kinase C, and ATP-sensitive potassium channel (K_{ATP} channel) in either the initiation and/or the maintenance phase of the ischemic preconditioning. The exact sequence of signalling events and the relationship between these post-receptor signalling molecules during both phases of preconditioning remain to be elucidated. Investigation of these signalling mechanisms should lead to the identification of new potential therapeutic targets in the treatment of patients with ischemic heart disease.

V. POTENTIAL CLINICAL USEFULNESS OF ADENOSINE ANALOGS

A. Clinical Evidence for Ischemic Preconditioning: Patient-oriented Investigations

1. In vitro Study on Human Cardiac Myocyte

Studies that provided the most direct evidence that preconditioning exists in humans ranged from *in vitro* experiments performed in cultured human cardiac ventricular myocytes [74] to various *in vivo* investigations in patients with known ischemic heart disease. In the *in vitro* study, human ventricular myocytes subjected to brief periods of ischaemia before 90 minutes of sustained ischaemia showed improved survival, compared to myocytes exposed to the 90-minute sustained ischaemia alone.

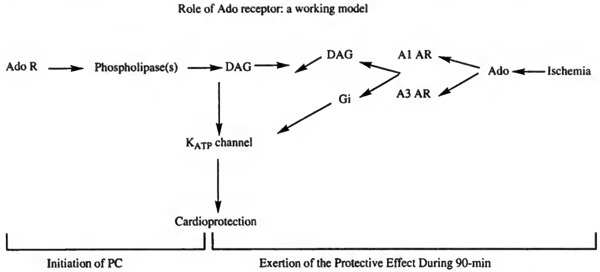

Role of Ado receptor: a working model

Fig. (4). A working model for the sequence of signaling events leading to the cardioprotective effect of ischemic preconditioning. In this model, the preconditioning process is divided into two phases, the initiation phase during which the process is triggered, and the maintenance phase during which the actual cardioprotection is exerted. The roles of adenosine receptor, phospholipases, diacylglycerol, protein kinase C and K_{ATP} channel are proposed.

2. *Investigation in patients undergoing CABG*

An example of clinical investigation which has implications for clinical practice, a study published in the Lancet in 1993 [76] described a preconditioning protocol in 14 patients undergoing cardio-pulmonary bypass. In this clinical protocol for preconditioning, 7 patients were subjected to two 3-minute periods of cross-clamping interspersed with 2 minutes of reperfusion before they were subjected to 10 minutes of cross-clamping for distal aorto-coronary anastomoses during coronary artery bypass grafting (CABG). The patients who received the two 3-minute periods of initial cross-clamping before the subsequent 10-minute cross clamping had significantly higher levels of adenosine triphosphate (ATP) – as assessed by analysis of myocardial biopsy specimens – than those who were subjected to the 10-minute cross-clamping alone.

3. *Studies on Patients with Acute Myocardial Infarction*

That ischaemic preconditioning might play an important role in humans became even more apparent after an interesting series of retrospective observations was made with regard to the effect of preinfarct angina on the outcome of patients with acute myocardial infarction (MI). In a study of 153 patients with their first Q-wave anterior wall acute MI, 100 had angina while 53 had no antecedent symptoms [77]. The presence of angina before acute MI was associated with a lower incidence of sustained ventricular tachycardia or fibrillation, cardiac rupture, lower in-hospital mortality, as well as lower peak creatine kinase (CK) than in patients without antecedent angina. No significant difference in collateral circulation was found between the two groups.

In the Thrombolysis in Myocardial Infarction (TIMI) 4 trial [78], 218 patients with acute MI and history of any prior angina experienced less in-hospital death, severe congestive heart failure (CHF) or shock than 198 patients with acute MI without prior angina. In addition, patients with any history of angina had smaller CK elevations and were less likely to develop Q-waves on their electrocardiograms (ECGs). Interestingly, this effect was again not dependent on angiographically detectable coronary collaterals, suggesting that the benefit of pre-infarct angina was indeed due to ischaemic preconditioning.

A similar study on 25 patients with their first anterior MI treated with thrombolysis [79] confirmed that patients with new-onset prodromal angina showed significantly lower CK-MB elevations and eventually suffered smaller infarcts (as assessed by the size of hypokinesis) than patients without prodromal angina. Again, no evidence of increased collateral circulation in patients with prodromal angina was found, further supporting the hypothesis of ischaemic preconditioning being directly responsible for the beneficial effect seen. The later TIMI 9 study [80] demonstrated a critical time dependency for the beneficial effect of pre-infarct angina. During a 30-

day follow-up after MI, patients who had angina less than 24 hours before MI had lower mortality, less CHF, and a lower incidence of shock or recurrent MI than patients without angina or with angina more than 48 hours before MI or never had angina before. This observation correlated well with the previous studies on animals showing a clear time-limit (window) for ischaemic preconditioning.

4. Ischemic Preconditioning and Patients Undergoing PTCA

In parallel to the retrospective studies showing the effect of angina on the outcome of subsequent MI, several prospective studies demonstrated the importance of ischaemic preconditioning in humans and the possibilities for future therapeutic interventions. Initial studies involved intracoronary balloon inflations, which were used to elicit the cardioprotective effect. In one of the earliest studies, repeated coronary artery occlusions during percutaneous transluminal coronary angioplasty (PTCA) were performed [61]. It was observed that during the second of two periods of occlusion of the left anterior descending (LAD) artery, patients experienced less anginal discomfort, and there was less ST segment shift, lower mean pulmonary artery pressure and less lactate production.

A subsequent study [62] demonstrated that during five successive balloon inflations within the LAD, there was a reduction of angina, ST-segment deviation, left ventricular (LV) filling pressure and impairment of LV ejection fraction. This study differed from previous ones by also demonstrating enhancement of recruitable collateral circulation in 10 of 17 patients during successive balloon inflations.

Recent research has introduced the concept of "pharmacological preconditioning" with the use of substances, e.g., adenosine, called "preconditioning mimetics" – to achieve the same cardioprotective effect but without the hazards of temporary coronary occlusion. The concept of preconditioning mimetics is based on the rationale that activation of adenosine receptor by the adenosine released during myocardial ischemia is responsible for initiating the preconditioning elicited during the first balloon inflation. Thus, one can mimic the salutary effect of the balloon inflation-induced preconditioning by giving an adenosine receptor agonist directly before any balloon inflation.

The success of such preconditioning mimetics was demonstrated in a study in which 15 patients received a 10-minute intracoronary infusion of adenosine [81] before the first PTCA balloon inflation. In patients who received adenosine, the subsequent 3 consecutive intracoronary balloon inflations produced smaller ST-segment shifts, compared to control patients who received only normal saline prior to PTCA. In addition, in control patients, the ST-segment shift in the intracoronary ECG was significantly greater during the first inflation than during the second and third inflations, consistent with ischaemic preconditioning. On the other hand, in the adenosine-treated patients, there was no differences in the ST-segment shifts during the three inflations. Even more importantly, the reduction in the ST-segment shift afforded by adenosine was greater than that afforded by ischaemic preconditioning.

Since protective effect of adenosine is a receptor-mediated phenomenon, it can be blocked by adenosine receptor antagonists (e.g., bamiphylline, aminophylline). In one study [82], bamiphylline - a selective antagonist of A_1 adenosine receptors - abolished the effect of adenosine on ST-segment shifts during PTCA. In another study [83], aminophylline, a nonselective adenosine receptor antagonist, inhibited ischaemic preconditioning – as assessed by analysis of the intracoronary ST-segment changes during angioplasty.

B. Ischaemic Preconditioning: Bridging Basic Research and Clinical Practice

Studies on ischaemic preconditioning have followed an established pattern of scientific discoveries; original observations made in a living organism are followed by *in vitro* studies attempting to elucidate the mechanisms of the observed phenomena. Once the mechanism is known, or at least a plausible hypothesis is postulated, clinical tests and trials follow, eventually bringing a new modality of medical treatment.

Although adenosine was used to successfully induce a preconditioning-like effect, there are a number of limitations which restrict the utility in a number of situations. Because of the extraordinary short life of adenosine (<10 seconds), adenosine needs to be administered as a continuous infusion in order to ensure a consistent occupancy and activation of the adenosine receptor. Adenosine can activate all four adenosine receptors; activation of the A_{2A} receptor can be potentially deleterious and counteract the myocyte protective effect mediated by A_1 or A_3 receptor. Activation of the A_1 receptor by adenosine may produce bradycardia or atrioventricular conduction block, especially if it is administered in the right coronary artery or the left-dominant circumflex artery. Adenosine itself is not orally available and can only be administered as an intravenous or intracoronary drug. Its availability to the cardiac adenosine receptor is limited by the extremely effective uptake system in the circulating blood cells and the endothelium. In this context, receptor-selective agents should prove to be superior to adenosine itself.

C. Potential Clinical Conditions Under which Pharmacological Preconditioning Agents May be Beneficial

a. unstable or stable angina – where administration of preconditioning mimetics could potentially reduce future infarct size or perhaps even prevent infarction altogether,

b. acute MI / reperfusion – on-going studies (e.g. HALT-MI) are examining the hypothesis that pharmacological preconditioning with adenosine receptor agonists could prevent/decrease the size of MI and extent of reperfusion injury,

c. PTCA – where the use of preconditioning mimetics could decrease/prevent CK leak, decrease potential myocardial injury and ultimately improve outcomes,

d. heart surgery (traditional CABG or "minimally invasive" procedures, like MIDCAB) – where preconditioning mimetics could minimize the ischaemic insult to the myocardium,

e. heart transplantation – where preconditioning mimetics could protect the donor heart peri-operatively, improve its future functioning and perhaps reduce the incidence of coronary artery disease in the transplanted heart.

The results of the above mentioned retrospective and prospective clinical observations on myocardial preconditioning (whether ischaemic or pharmacological) provide the initial encouraging clues to treat patients with ischemic heart disease. More studies in patients with ischemic heart disease are required before preconditioning can be applied routinely in clinical practice. Of particular interest would be tests on pharmacological preconditioning involving various preconditioning mimetics, like the already mentioned adenosine, K_{ATP} channel openers and/or other mediators postulated to be involved in the molecular mechanism of preconditioning. These agents could prove safer and easier to administer than intracoronary balloon inflations (ischaemic preconditioning). More importantly, these potentially novel pharmacologically preconditioning agents can be administered under clinical conditions in which balloon inflation is not part of the therapeutic procedure. Potential clinical situations during which these agents may be beneficial are discussed below.

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Strategies and Progress Towards the Ideal Orally Active Thrombin Inhibitor

J. B. M. Rewinkel*[1] and A. E. P. Adang[2]

[1]Department of Medicinal Chemistry, [2]Lead Discovery Unit, Chemistry Group, NV Organon, Research and Development, P.O. Box 20, 5340 BH Oss, The Netherlands

Abstract: Thrombin plays a key role in the control of thrombus formation, for which reason its inhibition has become a target for new antithrombotics. Important issues in the profile of the ideal thrombin inhibitor are: potency, selectivity, oral bioavailability, half-life in the circulatory system and safety. Although many potent direct inhibitors of thrombin have been discovered, most of these inhibitors lack sufficient oral bioavailability. This is often associated with the presence of highly basic functionalities such as guanidine or amidine. These basic functionalities in the P_1 moiety are preferred by thrombin and are present in the first generation of thrombin inhibitors. Recently, several orally active direct thrombin inhibitors have been disclosed. Most of these inhibitors originate from leads of the first generation. Two major optimization strategies could be identified to further improve these leads: A: maintain the highly basic P_1 moiety and compensate its negative effects, and B: reduce the basicity of the P_1 moiety and compensate for the decrease in inhibitory activity. The progress made using these strategies is evaluated. In addition, screening large sets of compounds yielded new structures that provide useful starting points for optimization. The optimization strategy used to convert leads from screening into potent orally active thrombin inhibitors is also be evaluated.

INTRODUCTION

Thromboembotic diseases are the number one cause of illness and death in developed societies for a long time [1]. New and better antithrombotic drugs may therefore contribute significantly to an improved health state of the population. A few years ago Sixma and de Groot discussed various criteria for the ideal anti-thrombotic drug [2]. In their view these criteria are: inhibition of thrombosis without affecting haemostasis, a long half-life, oral bioavailability, no serious side-effects and a large therapeutic range. In the search for new antithrombotics the central role of thrombin (IIa) in haemostasis and thrombosis has made it a prominent target. Research aiming at orally active thrombin inhibitors has focused on finding ligands for the active site, the so-called direct thrombin inhibitors. Inhibitors for other binding sites in thrombin such as the anion binding exosite are excluded in this review. Several potent inhibitors of thrombin have been known for almost twenty years [3 - 7] and crystallographic data of the thrombin-inhibitor interactions are available for the lastdecade [8 - 10]. In addition, the research interest in direct thrombin inhibitors has been intense for a long period as illustrated by the recent review of thrombin inhibitors in the patent literature [11]. Moreover, the usefulness of direct thrombin inhibitors as antithrombotics has been demonstrated in recent clinical trials with i.v. formulated direct thrombin inhibitors [12]. Despite all this, the thrombin inhibitor that fulfills all the criteria mentioned above still remains elusive. Especially the introduction of the proper pharmacokinetic properties has been problematic. Recently, several low molecular weight (LMW) direct thrombin inhibitors that are orally active have been disclosed. Despite the great chemical heterogeneity of these orally active compounds, the optimization from LMW lead compounds to orally active drugs can be grouped in a limited number of strategies. This review describes these strategies and the way they were applied and evaluates the progress made towards the ideal antithrombotic drug.

THROMBIN'S ROLE IN HAEMOSTASIS AND THROMBOSIS

Since the role of thrombin in haemostasis and thrombosis and other biological roles has been

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*Address correspondence to this author at the Department of Medicinal Chemistry, NV Organon, Research and Development, P.O. Box 20, 5340 BH Oss, The Netherlands; e-mail: j.rewinkel@organon.oss.akzonobel.nl

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reviewed in detail elsewhere, the present article will only address the role of thrombin in haemostasis and thrombosis briefly [5, 13].

In the coagulation cascade thrombin is the final key enzyme. It cleaves fibrinogen into fibrin and activates factor XIII to cross-link soluble fibrin to form a fibrin matrix for the developing blood cloth. In addition, thrombin amplifies its generation by activating factors V and VIII (positive feedback) but also inhibits its generation by activation of the protein C pathway (negative feedback). This balance between pro-coagulant and anti-coagulant mechanisms is important in the control of haemostasis. Thrombosis can be regarded a condition where improper regulation of the haemostasis mechanisms resulted in the formation of intravascular clots which may lead to tissue damage or cell death due to inadequate blood flow.

THROMBIN'S ACTIVE SITE

Thrombin is a member of the trypsin family of serine proteases. Serine proteases are proteolytic enzymes which all contain the characteristic serine-histidine-aspartate catalytic triad (Ser^{195}, His^{57} and Asp^{102} in thrombin [14]). The hydroxyl group of serine is activated in the presence of the other two members of the triad and during the proteolytical process acts as a nucleophile targeting the scissile amide bond of the substrate. In addition, the developing negative charge of the tetrahedral intermediate is stabilized by backbone amides creating the so-called oxyanion hole (the backbone of Gly^{193} and Asp^{194} in thrombin). Proteases of the trypsin family may further be subdivided according to the amino acid after which they prefer to cleave; the so-called P_1 amino acid. In the largest subfamily a basic amino acid, arginine or lysine, is preferred at the P_1 position. This subfamily includes, amongst others, the digestive enzyme trypsin, the blood coagulation enzymes thrombin, factor VII, factor IX, factor X, factor XII and kallikrein, the anti-coagulant protein C, and the thrombolytic enzymes urokinase, plasmin and tissue plasminogen activator. Thrombin has a natural preference for arginine at the P_1 position as demonstrated by the highly conserved arginine in its natural substrates such as fibrinogen, factor V, factor VIII and factor XIII.

The structural interaction of natural substrates like fibrinogen and of several inhibitors with thrombin have extensively been characterized using crystal structures [8 - 10, 15]. Although thrombin has several high affinity binding sites (anion binding exosite, acive site, heparin binding site), the search for orally active inhibitors has focused on the cleavage site, usually called the active site. Some structural features of the active site are schematically depicted in Fig. (**1**). The already mentioned catalytical triad is located on the right side of Fig. (**1**). Thrombin's preference for arginine results from the topography of the specificity pocket (S or S_1 pocket). The top of this pocket is hydrophobic and at the bottom the carboxylic side chain of Asp^{189} is situated. By forming an acid - base interaction the carboxylate of this Asp residue provides the specificity for basic amino acids at the P_1 position. Adjacent to the S-pocket are two hydrophobic pockets: a proximal pocket (P pocket) and a distal pocket (D pocket) to the catalytic center. The P pocket is also called the S_2 pocket, it is of moderate size and it binds the P_2 amino acid valine of fibrinogen (Fig.(**2**)). The D-pocket is larger and is also called the aryl binding site since this site is often occupied by an aryl moiety. In fibrinogen this pocket is occupied by phenylalanine, which is actually the P_9 amino acid in this case. Furthermore, below the hydrophobic pockets Gly^{216} is depicted which, usually makes two hydrogen interactions in a anti-parallel manner and is sometimes called the S_3 site. For fibrinogen this interaction is with the P_3 amino acid glycine.

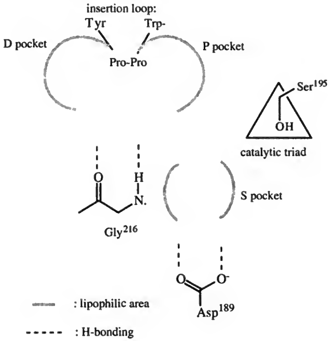

Fig. (1). Schematic representation of thrombin's active site

Some typical structural features of thrombin's active site are well exemplified by comparison of its active site with the one observed in trypsin, a digestive serine protease with low substrate

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specificity. First, located in the S pocket is amino acid 190 which is an alanine in thrombin and a serine in trypsin. This indicates that the S-pocket in thrombin is slightly larger and more hydrophobic than the one in trypsin. Second, thrombin has an extra loop, the Tyr-Pro-Pro-Trp insertion loop at position 60 between the P and D pocket, not present in trypsin. These differences derived from the 3-D structural data have been exploited to obtain more selective thrombin inhibitors.

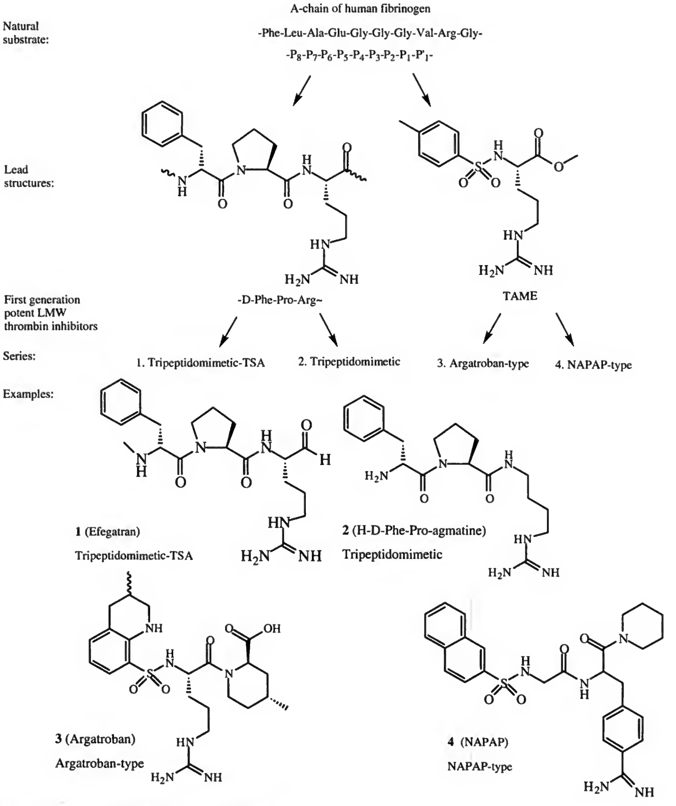


Fig. (2). Series of LMW-leads derived from fibrinogen.

FIRST GENERATION OF LMW THROMBIN INHIBITORS

The preference for arginine at the P_1 position of fibrinogen was the starting point for the design of the first generation of LMW thrombin inhibitors. Based upon their chemical structures and the nature of the interactions with thrombin these fibrinogen derived inhibitors can be ordered into four major classes (Fig. (**2**)).

The first two classes are the result of investigations in the structure-activity-relationship of the peptide sequence in fibrinogen that immediately precedes the scissile bond. This resulted in the identification of the active tripeptide sequence D-Phe-Pro-Arg. In the first class the scissile amide bond at the C-terminus of arginine was replaced by an electrophile, the transition state analogue (TSA), which forms a complex with Ser^{195} of the catalytic triad. This TSA-Ser^{195} complex is mimicking the tetrahedral intermediate (transition state) and results in an increased binding affinity. We have named this class the tripeptidomimetic-TSA thrombin inhibitors. One of the oldest examples of this class is efegatran (Me-D-Phe-Pro-Arginal, **1**) where the electrophile is an aldehyde group that forms a hemiacetal bond with Ser^{195} [16]. In the second class, the tripeptidomimetics, the D-Phe-Pro moiety and mimics thereof are attached to a arginine moiety lacking its carboxylate. The first compound in this class was H-D-Phe-Pro-agmatine (**2**) [17]. The interactions of **2** with thrombin are very similar to the ones observed for tripeptidomimetic-TSA compound **1** (Fig. (**3**)) [18].

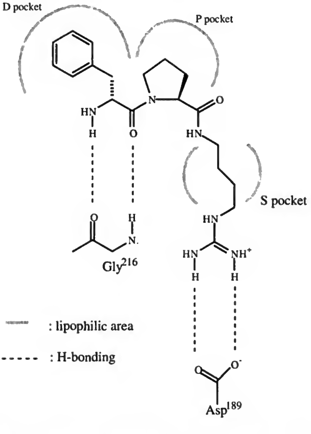


Fig. (3). Schematic presentation of the binding mode of H-D-Phe-Pro-agmatine (**2**) to thrombin.

Also the last two classes of the first generation of inhibitors were inspired by the preference of thrombin for arginine. In the third class Nα-tosylarginine methyl ester (TAME) was varied in such way that the ester was exchanged for tertiary amides [19]. Optimization of the arylsulfon amide and tertiary amide yielded the potent thrombin inhibitor argatroban (**3**). We have called this class the argatroban-type series. This series can broadly be characterized as inhibitors in which the carboxylate of the central amino acid is functionalized as a tertiary amide and the α-position is substituted with an aryl sulfonamide moiety.

The fourth class used 4-amidinophenylalanine as a mimic for arginine. Again variation of the Nα and carboxylate substituent yielded a potent inhibitor: NAPAP (**4**) [20]. We have called this small class the NAPAP-type series. This class can broadly be characterized as inhibitors in which the carboxylate of the central amino acid is functionalized as a tertiary amide and the α-position is substituted with a N-aryl sulfonyl amino acid.

Comparison of the binding mode of argatroban and NAPAP, depicted in a schematic way in Fig. (**4**), reveals some remarkable similarities and differences [9]. In both cases the basic moiety interacts with Asp^{189} of the S pocket, the tertiary amide substituent occupies the P pocket and the aryl moiety attached to the sulfon amide occupies the D pocket. However, the central moiety has a quite different binding pattern in these two inhibitors. The central amino acid moiety of argatroban has the L-stereochemistry and interacts via antiparralel hydrogen bonding with Gly^{216} whereas in the central dipeptide moiety of NAPAP the phenylalanine part is a D-amino acid and the glycine has the hydrogen bonding interactions with Gly^{216}.

In the remainder of this paper the peptide nomenclature is applied to the inhibitors. The moiety binding to the S pocket we refer to as P_1 moiety, the P pocket binding moiety as P_2 moiety and the D pocket binding moiety as P_3 moiety. In the first generation leads the P_1 moiety contains the highly basic moiety. The P_2 moiety is in series 1 and 2 the proline moiety and in series 3 and 4 the

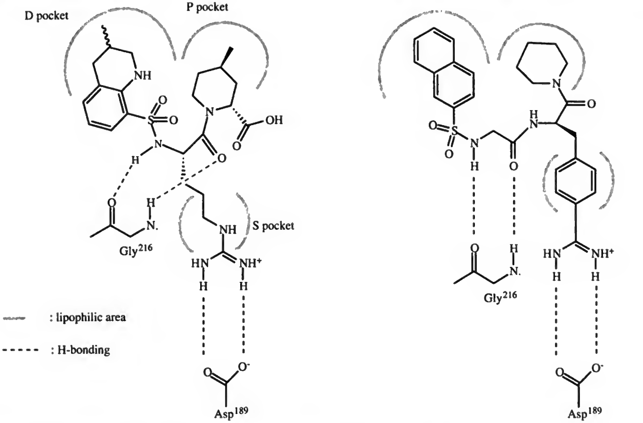


Fig. (4). Schematic presentation of the binding mode of argatroban (3) and NAPAP (4) to thrombin.

tertiairy amide moiety. The P_3 moiety is in series 1 and 2 the D-phenylalanine moiety and in series 3 and 4 the aryl sulfonamide moiety.

Although the structures of the inhibitors in the four classes described above have different structures they still have some common features. They all have an amino acid framework that contains a highly basic guanidine or amidine function, have several hydrogen bonding groups and have a tripeptide size with a molecular weight above 350 Dalton. To obtain the optimal antithrombotics the optimization strategies should address issues that can roughly be divided in antithrombotic issues and pharmacokinetic issues.

ANTITHROMBOTIC ISSUES

The efficacy and safety of a drug are always important issues. Safety in antithrombotic drugs means that the compound should not be toxic in any way but also that bleeding when it occurs can be controlled [21]. The toxicity of compounds is often compound specific and will not be discussed here. However, bleeding is an significant topic closely related to the beneficial effects of antithrombotics since they alter the haemostatic balance. To minimize the bleeding risk inhibitors should be selective for thrombin and not inhibit other serine proteases especially not the ones involved in the anticoagulant and thrombolytical pathways.

Although a good inhibitory activity of thrombin in *in vitro* assays is a good starting point, caution must be applied to extrapolate the inhibition values obtained with chromogenic substrates into physiological situations. Taparelli and co-workers revealed that not only the Ki value and selectivity but also the kinetics of thrombin inhibition influences the efficacy in *in vivo* antithrombotic models [5, 22]. They postulated that slow binding borate based tripeptidomimetic TSA inhibitors are not as efficacious as might be expected based on their Ki values because these inhibitors do not inhibit fast enough. It was concluded that the binding behavior of the inhibitor with thrombin must be rapid. Furthermore, also the actual concentration of unbound inhibitor in plasma is of importance. Researchers at Merck reported that within a series of thrombin inhibitors the increased lipophilicity and the associated increased plasma protein binding had detrimental effects on the performance in *in vivo* antithrombotic models [23]. Also von der Saal reported that the coagulation times did not correlate with the Ki values and compound lipophilicity needed to be considered together with thrombin inhibitory potency [24].

PHARMACOKINETIC ISSUES

Following oral administration of a drug several processes can be distinguished: - absorption from the intestine, - distribution from the plasma into organs and tissues and - elimination by metabolism and/or excretion. Absorption from the intestine has been reviewed extensively[25, 26]. In order to be absorbed from the intestine compounds should have some aqueous solubility and be stable in the GI tract. Compounds can be absorbed by four different pathways across the intestinal epithelium: transcellular route (passive absorption), carrier-mediated route (active absorption), paracellular route and transcytosis route. The latter one is a process not well-understood yet and is probably not suited for the absorption of LMW-thrombin inhibitors. The paracellular route is probably also not suited for LMW-thrombin inhibitors since this route usually involves small compounds with molecular weight smaller than 200 Dalton. Active absorption is observed for di- and tri-peptide like compounds and involves the recognition and transport by the small-peptide carrier. According to the recently described pharmacophore di- and tripeptide like compounds suited for this carrier should have: - a free carboxylic acid group, - a peptide bond and - no second negative charge [27]. Taking these properties in consideration leads of the first generation, tripeptide-like, might become suited for active transport after some optimization.

Passive absorption is the most important route for drug absorption across the intestine. In this process a molecule dissolved in the aqueous environment in the intestine has to pass the lipophilic membranes. Efficient passive absorption reflects the ability of a molecule to cope with the desolvation energy involved in this process. Therefore several physicochemical parameters involved in the desolvation energy, such as: charge and hydrogen binding capacity, will influence absorption. In literature several descriptors are being used to predict passive absorption. The rule of 5 described by Lipinski predicts that poor absorption is likely when there are more than 5 hydrogen bond donors, more than 10 hydrogen bond acceptors, the molecular weight (MW) is greater than 500 and the calculated Log P is greater than 5 [28]. Another interesting descriptor to predict the passive oral absorption of drugs is the dynamic polar molecular surface area [29]. In Table 1 several properties present in the first generation of direct thrombin inhibitors are compared with the properties required for passive and active absorption. This table clearly indicates the hurdles that drug hunters must take to achieve oral bioavailability in their thrombin inhibitors. Moreover, a low level of oral bioavailability will not be good enough. As Menear postulated in a recent review: 20% bioavailability would be a requisite to prevent large inter-patient variability in light of the expected narrow therapeutic window [7].

The second process in pharmacokinetics is the distribution of the drug across various tissues and organs. The target organ of thrombin inhibitors is the plasma and as already described above, binding to proteins has a negative impact on the antithrombotic potency. Consequently, if these findings are translated into pharmacokinetic parameters this would mean that the distribution volume should be small. In addition, a large distribution volume would mean that a large amount of an antithrombotic compound has to be administered to achieve plasma levels at a therapeutic range. A possible depot formation would result in a complex kinetic profile. Therefore, design strategies should aim at moderate lipophilicity and good aqueous solubility.

The third process in pharmacokinetics is elimination by metabolism and/or excretion. A method to reduce the influence of elimination is to design compounds with a large distribution

Table 1. Hurdles for Oral Bioavailability of the First Generation Thrombin Inhibitors

Thrombin inhibitors	Passive diffusion	Active absorption
MW > 350	low MW lipophilic	di/tripeptide size di/tripeptide like
oriented H-bonds (with Gly^{216} and Asp^{189})	minimum H-bonds	H-bonds allowed
guanidine / amidine functionality preferred	no strongly basic functionality preferred	basic functionality allowed

volume. As indicated above this strategy is not a desirable one for antithrombotic drugs. Consequently, the ideal antithrombotic drug should have a small clearance in order to achieve a long half life in combination with a small volume of distribution. The elimination of drugs is complex since it involves several organs and mechanisms and no clear guidelines for reducing the rate of elimination are given in the literature. Hepatic clearance of drugs is not only of great importance for both the clearance of compounds from the system but also for the oral bioavailability, the so-called first pass effect.

Optimization strategies have to deal with all these pharmacokinetic issues raised, some of them being specific for the type of drug.

DRUG OPTIMIZATION STRATEGIES

A typical feature of the LMW-thrombin inhibitors of the first generation is the presence of a highly basic guanidine or amidine moiety. However, these highly basic groups probably also form a major hurdle in the improvement of the pharmacokinetic profile of these inhibitors. Two strategies have been applied to overcome this hurdle (Fig. (**5**)). In the first strategy the P_1 moiety containing a highly basic group is maintained, thereby keeping thrombin's preferred interaction in the S pocket. The P_2-P_3 moiety of the lead is either explored with P_2-P_3 isosters of a less peptidic character or scanned for a free site that can be modified (often introduction of a carboxylate) without negatively affecting the anti-thrombin activity. Once such P_2-P_3 isoster or free site is found small modifications are made in the rest of the molecule to optimize the properties. In the second strategy the highly basic P_1 moiety is replaced by moieties of attenuated basicity that still leave some inhibitory activity and improve the non-inhibitory properties of the lead. Thereafter, efforts are devoted to rebuilding the anti-thrombin activity. Again, the last step is making small chemical modifications in the rest of the molecule to obtain the optimal profile. Both strategies are fueled by crystallographic data of the thrombin-inhibitor interaction.

The strategies described above start with LMW leads derived from fibrinogen and all these leads have an amino acid framework that contains a highly basic function, have several hydrogen bonding groups and have a molecular weight above 350 Dalton. Screening, however, presents the

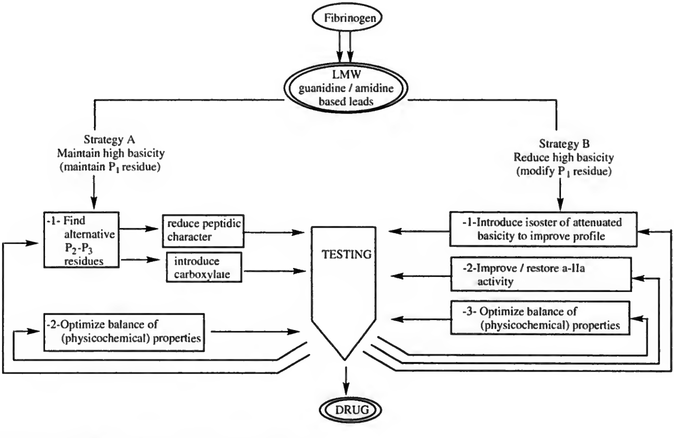

Fibrinogen
LMW guanidine / amidine based leads
Strategy A
Maintain high basicity
(maintain P_1 residue)
-1- Find alternative P_2-P_3 residues
reduce peptidic character
introduce carboxylate
-2-Optimize balance of (physicochemical) properties
TESTING
Strategy B
Reduce high basicity
(modify P_1 residue)
-1-Introduce isoster of attenuated basicity to improve profile
-2-Improve / restore a-IIa activity
-3- Optimize balance of (physicochemical) properties
DRUG

Fig. (5). Optimization strategies for thrombin inhibitors.

opportunity to radically depart from amino acid based leads and to deliver novel leads in the search for the ideal thrombin inhibitor.

In the following sections we want to describe the results obtained in the four classes of fibrinogen derived LMW leads according to the optimization strategies applied. In addition, the results from leads obtained from screening and their optimization will be evaluated. Finally, we summarize the progress made towards the ideal antithrombotic.

SERIES 1. TRIPEPTIDOMIMETIC-TSA COMPOUNDS

This series concerns the tripeptide inhibitors derived from one of the natural substrates of thrombin, fibrinogen. At the C-terminus of these tripeptides an electrophilic moiety has been incorporated. This electrophile binds to Ser^{195} of the catalytic triade in thrombin's cleavage site thereby mimicking the transition state of amide bond hydrolysis and adding considerably to the binding affinity. In general terms these compounds can be described as *in vitro* highly potent peptidic inhibitors. In addition, some concerns exist on their relatively low selectivity over related proteases and their slow binding kinetics that often is observed [22, 30]. These factors are thought to complicate progress to *in vivo* efficacious and safe antithrombotics. The most important series of transition state analogs will be described and emphasis is on the design of compounds in relation to the points raised above.

1a. Aldehydes

Optimization Strategy A: Maintain High Basicity

Detailed optimization studies on the active site binding part of fibrinogen resulted in the D-Phe-Pro-Arg sequence as an inhibitory entity for thrombin [31]. Bajusz and co-workers introduced an aldehyde at the C-terminus and by doing so prepared the first transition state analog (TSA) inhibitor of thrombin: compound **5** [32]. The N-methyl variant **1** was found to be more stable and was evaluated in clinical trials by Eli Lilly. Some oral bioavailability in rats was reported since (12%) [33] but further development of this compound was not observed [33]. Eli Lilly continued on this track and kept the arginine aldehyde (arginal) constant while systematically changing the D-Phe-Pro (P_3-P_2) part. An important analog that was synthesized is compound **7**. This analog is very potent *in vitro* with a reported Ki of 3.3 nM and, more interestingly, 54% oral bioavailability in rats [34]. Besides an isolated report by BMS [35] on arginal derivative **8**, Eli Lilly and Corvas have been the major players in this area. Compound series **9** to **12** disclosed by Corvas nicely illustrates a gradual shift towards non-peptidic P_3-P_2 units attached to the arginal group [36 - 39]. Interestingly, these non-peptide moieties still are capable of interacting with Gly^{216} in thrombin through hydrogen bonds [37, 38]. This interaction between thrombin and its inhibitor results in an antiparallel ß-sheet formation reminiscent of the binding of fibrinogen.

Several of the Corvas compounds have been reported to have oral bioavailability in animals and have progressed towards the clinic. Trypsin selectivity improved considerably when amidinopiperidines were incorporated in stead of a guanidine but lower oral activities were the result [40]. A possible explanation may be that arginal compounds are transported over the intestinal boundary in its cyclic hemiaminal form. Available literature and drug databases suggest that no compounds belonging to this class are currently undergoing clinical evaluation.

In this sub-class of aldehydes no examples were reported of an optimization strategy in which the high basicity of the P_1 moiety was reduced.

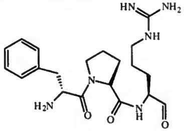
1b. Boronic Acids

Chronologically, boronic acid was discovered as a transition state mimic after the aldehydes. The lead compound originated from DuPont and was called DuP714, compound **13** [41]. It appears that boronic acids and its prodrugs like pinadiol esters are the most powerful TSA one can find for thrombin. As a consequence, substantial departures from the structure of **13** are allowed as long as the boronic acid moiety is left intact. Both strategies A and B have been tried in order to make an improved DuP 714 analog without its side effects like hypotension and immune reactivity [42].

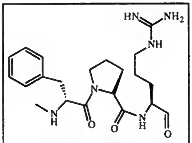
Optimization Strategy A: Maintain High Basicity

Compounds **14** and **15** are examples where modification of the P_3-P_2 peptide moiety resulted in improved compounds. Especially Servier's compound **14** that entered clinical trials was reported to have no immune response and 60% p.o.

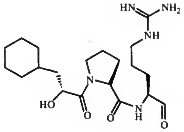
Table 2. Aldehydes



5 (GYKI-14166)



1 (GYKI-14766, efegatran)



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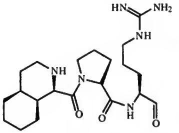

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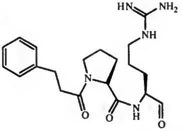

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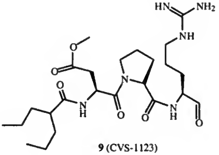
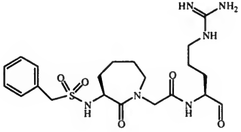
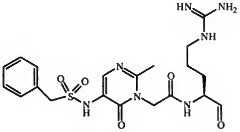


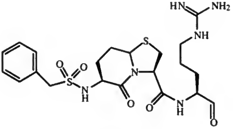
9 (CVS-1123)



10 (CVS-1778)



11 (CVS-2038)

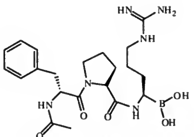


12 (CVS-1862)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
5	0.032*			
1	0.040*	12		Phase I clinic
6	0.157*			
7	0.0033	54		
8	0.14			
9	0.00048			monkey F = 36%
10	0.00054		66	20 mg/kg p.o. dog, Phase I clinic stopped
11	0.0023**			
12	0.016**			

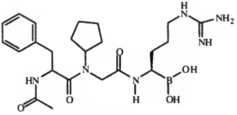
* IC_{50} Thrombin Time ** IC_{50} chromogenic assay

Table 3. Boronic Acids

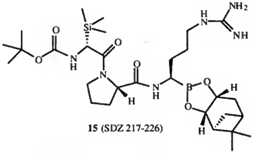


13 (DuP-714)

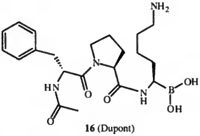
Strategy A:
Maintain high basicity



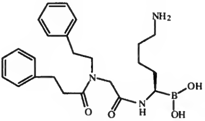
14 (S-18326)



15 (SDZ 217-226)

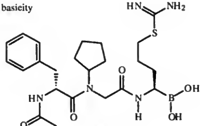


16 (Dupont)

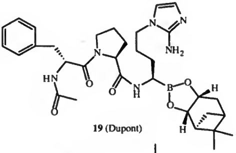


17 (Dupont)

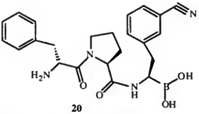
Strategy B:
Reduce high basicity



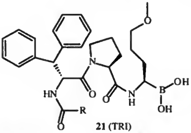
18 (S-18821)



19 (Dupont)



20

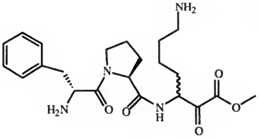


21 (TRI)

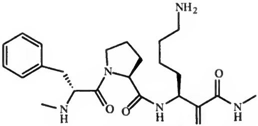
Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
13	0.000041			hypotension, immunoresponse
14	0.004*	8	60	Phase I clinic, stopped
15	0.000041			
16	0.00024			
17	0.00012		+/-	2xAPPT at 5 mg/kg
18	0.00059*			more selective than **13**
19	0.0017			
20	0.00048			
21	0.014			

* IC_{50} chromogenic assay

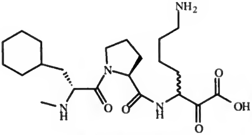
Table 4. Keto-carbonyl Compounds



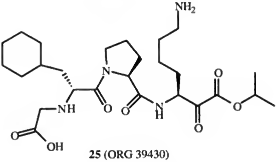
22 (BMS 181,412)



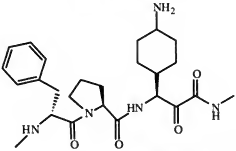
23 (Merck)



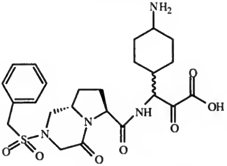
24 (Ferring /Astra)



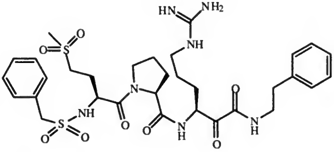
25 (ORG 39430)



26 (Merck, L-370518)



27 (Biochem Pharma)



28 (CVS-1347)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
22	0.032*			not stable, slow binding
23	0.0028	12-15	low	slow binding
24	0.007**			slow binding
25	0.020***	32	71	$T_{1/2}$ 58 and 108 min resp.
26	0.00009	<10	<10	
27	0.00009			
28	0.00013			

* IC_{50} chromogenic assay. **IC_{50} thrombin time, *** calculated K_i

activity in dogs [43, 44]. Compound **16** and **17** from Dupont contain a lysine in stead of an arginine at the P_1 position [42, 45]. With a pKa of around 10.5 it is still relatively basic and interestingly enough even a simplified lysine analog like **17** is still a subnanomolar inhibitor of thrombin.

Optimization Strategy B: Reduce High Basicity

Other peptides with modified guanidine moieties, like the isothiourea group in **18**, were disclosed by researchers at Servier. They compared **13** with **18** and concluded that **18** was equipotent and much more selective towards related proteases, especially those involved in fibrinolysis [46]. The amino imidazole group in compound **19** is a true isoster for a guanidine with a pKa of around 9.8 [47]. Loss of 1-2 orders of magnitude in inhibitory potency is acceptable in this series because the starting point was an inhibitor with a Ki of 41 picomolar.

To stress the uniqueness of the boronic acid class, researchers replaced the guanidine group with non-basic moieties like the *meta*-cyanophenyl in **20** and the methoxy propyl in **21** [48, 49]. The difference in activities between these two compounds might be due to more favorable hydrophobic interactions in the upper part of the specificity pocket with the aromatic ring of compound **20** and a hydrogen bond interaction between the cyano group of **20** and Gly^{219} of thrombin.

The current understanding is that these peptidic compounds with a reactive boronic acid moiety are no longer being persued any further.

1c. Keto-carbonyl Compounds

Interest in this more recent type of TSA finds its origin in a paper by Iwanowicz describing compound **22** [50] and the isolation and characterization of cyclotheonamides from marine sponges [51]. Cyclotheonamide A and B are cyclic pentapeptide like structures with an arginine ketoamide as the most striking characteristic. Apparently nature has invented its own transition state analog structures for protease inhibition. Although in some other areas of protease inhibitor design the keto-carbonyl TSA had proven to be *in vitro* effective it still opened up a new area in the design of thrombin inhibitors [52].

Optimization Strategy A: Maintain High Basicity

Researchers at Merck, Ferring/Astra and Organon investigated the potential of lysine keto-carbonyl derivatives (**23**, **24** and **25** respectively). In 1995, a series of publications emerged describing potent thrombin inhibitors based on the lysine keto-carbonyl structure (e.g. [53, 54]). Low oral bioavailabilities were reported for compound **23** and related compounds and the observed slow binding characteristics of compounds **23** and **24** was seen as a complicating factor. As a result both Merck an Astra seemed to have moved away from this area towards non-transition state compounds.

At Organon we continued this approach and reported in 1998 compound **25** that showed high oral bioavailabilities in rats and dogs, respectively 32 and 71%. It was also mentioned that the antithrombotic potency was improved compared to argatroban even though both compounds had Ki values of 20-30 nM. Introduction of the carboxy methyl group at the N-terminus was the key to improved intestinal absorption [55]. The aminocyclohexyl moiety disclosed by Merck and incorporated in **26** and **27** gave improvements in Ki and selectivity but worsened the oral bioavailability [56, 57]. Similarly, compound **28** with a modified N- and C-terminus showed high *in vitro* potency but no promising *in vivo* behavior [58].

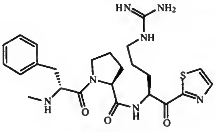
1d. Ketoheterocycli

Optimization Strategy A: Maintain High Basicity

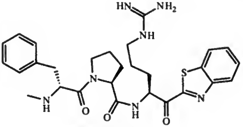
In more recent years arginine-(2-thiazolyl) and related structures appeared in the scientific and patent literature. The first published compounds originated from Johnson & Johnson: **29** and **30**, [59, 60]. Closely related structures from Corvas and MSK, **31** and **32** respectively, appeared in literature in the same time frame [61, 62]. In line with previous reports on arginine - TSA compounds it came as no surprise that these compounds are not only very potent (~1 nM) thrombin inhibitors but also prone to side effects. Furthermore, again only very low oral bioavailability was reported for **29** [63].

Several groups were aiming at further reduction of the peptidic character of the P_3-P_2 moiety. Joint investigations at BioChem Therapeutic and Parke-Davis resulted in non-peptides like compound **33** [64]. The latter compound was a selective and potent inhibitor of thrombin but lacked significant activity after oral administration. A similar approach at Molecumetics resulted in the potent but not selective thrombin inhibitor **34** [65]. The oral bioavailability of this compound has not been reported.

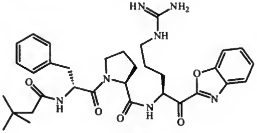
Table 5. **Keto-heterocycli**



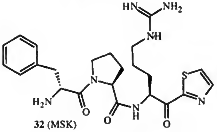
29 (RWJ-50026)



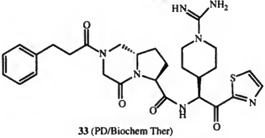
30 (RWJ-50353)



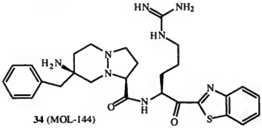
31 (Corvas)



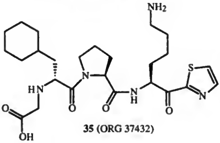
32 (MSK)



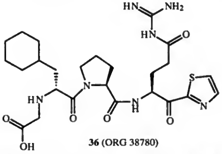
33 (PD/Biochem Ther)



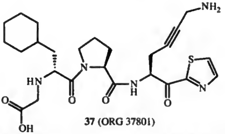
34 (MOL-144)



35 (ORG 37432)



36 (ORG 38780)



37 (ORG 37801)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
29	0.0036	1		side effects
30	0.00019			
31	0.068*			
32	0.0015*			
33	0.001	low		trypsin selective, 30mg/kg po rat
34	0.00066			
35	0.020	23	38	
36	0.00055**			
37	0.009**			

* IC_{50} chromogenic assay, ** calculated K_i

In this series of TS-analogs compound **35** prepared at Organon is the exception concerning oral bioavailability [55]. Again, as with keto-ester **25**, a carboxylate at the N-terminus of a lysine containing TSA resulted in a potent orally bioavailable antithrombotic compound. Design was guided by the knowledge of other polar, peptidic and orally active drugs like lisinopril and cephalexine. These comparisons proved very useful in the final design cycles where a balance of physicochemical properties was obtained.

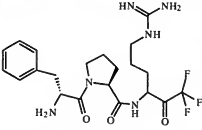
Optimization Strategy B: Reduce High Basicity

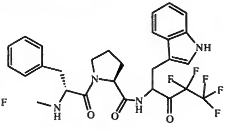
At Organon we prepared compounds **36** and **37** in which the less basic acylguanidine (pKa = 7.6) and butynylamine (pKa = 8.3) were incorporated as P_1 residues. In both cases potent thrombin inhibitors were obtained [66, 67].

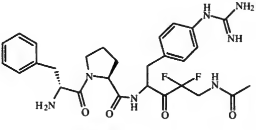
1e. Miscellaneous

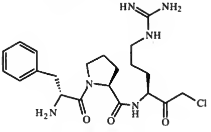
So far the four main approaches in TSA design have been described in more or less chronological order. In Table 6 a summary is given of other TSA structures that appeared as isolated reports throughout the last twenty years [68-75]. It shows the diversity of structures that are able to mimic the transition state of amide bond hydrolysis. Interesting enough both highly basic P_1 moieties and completely neutral P_1 analogs (**39** and **44**) were combined with a TSA moiety indicating the

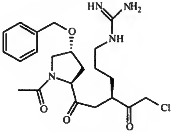
Table 6. Miscellaneous TSA-peptidomimetics

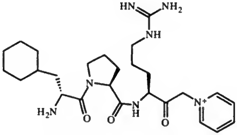

38 (MDL-73756)

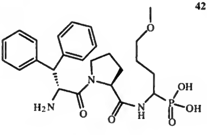

39 (MDL)

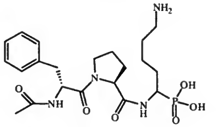

40 (MDL)


41 (Dupont)


42 (RPR)


43 (BRI)


44 (TRI)


45 (Dupont)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
38	< 0.001			
39	0.247			
40	0.070			
41	0.0015			PPACK, side effects, poor PK
42	0.0009*			more selective towards FXa
43	0.0013			
44	0.0048			Diphenyl phosphonate prodrug
45	6.5*			Diphenyl phosphonate prodrug

* IC_{50} chromogenic assay

tolerance of thrombin for active site ligands. Although these diverse TSA structures serve their use in protease inhibitor design, no progress towards a p.o. bioavailable drug candidate was ever reported.

In conclusion, despite the large number of potent thrombin inhibitors in this tripeptidomimetic-TSA series limited progress is observed towards the ideal antithrombotic. The most promising strategy in this series appears to be strategy A: maintain the highly basic moiety. This strategy resulted in several orally bioavailable compounds. Due to low selectivity, slow binding kinetics and complex chemistry most groups have left this series. In the aldehyde and boronic acid sub-series no progress is observed during the last years. In the keto-carbonyl and ketoheterocycli sub-series some promising new compounds such as **25** and **35** have emerged that approach the criteria for the ideal antithrombotic drug.

SERIES 2. TRIPEPTIDOMIMETIC COMPOUNDS

The limited progress towards orally bioavailable thrombin inhibitors in the TSA area in combination with the perceived liabilities described above resulted in a revitalization of compounds lacking the C-terminus of the arginine residue. Again we owe this starting point to Bajusz and co-workers who already described agmatine derivative **2** in 1982 [17, 76]. The penalty for deleting the TSA moiety was 2-3 orders of magnitude loss of inhibitory potency. As exemplified in the following sections, subsequent design cycles resulted in complete restoration of inhibitory potency thereby showing the power of lead optimization.

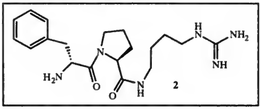
Optimization Strategy A: Maintain High Basicity

Several potent inhibitors resulted from the strategy in which the highly basic P_1 moiety was maintained and an additional carboxylate was introduced in the peptidic P_3-P_2 structure (Table 7). Inogatran (**46**) and Melagatran (**47**) are drug candidates that resulted from optimization programs at Astra. Inogatran was reported to be orally bioavailable only at higher dosages and still had a trypsin activity in the same range as its thrombin activity [77]. As a follow-up Astra disclosed Melagatran, a benzamidine-based analog of **2** and **46**, with improved potency and oral activity. Again, in a short period of time (1995-1996) not only Astra published its patent application on Melagatran and its analogs, but also Eli Lilly, Mitsubishi and BASF disclosed related non-TSA benzamidine series [78 - 81]. Both Astra compounds have advanced into clinical trials and first reports indicate that **47** is at least as effective as heparin in patients with DVT when given as an infusion for 4-6 days [82]. No reports have been made public on possible positive effects after oral administration to humans. The amidino-indole derivative **48** is another example of an orally bioavailable thrombin inhibitor combining a basic moiety with a carboxylate [83]. In this case the D-amino acid-Pro (P_3-P_2) moiety has been replaced by an isoster. Pfizer reported on an interesting switch in substituents at P_3: a N-alkylated aspartate, **49** [84].

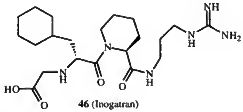
Compounds **50 - 55** are examples of highly basic moieties at P_1 with modifications at P_3-P_2 different from the basic-carboxylate design. Eli Lilly described a series of N-terminal substituted tripeptidomimetics such as sulfonamide **50** which showed some oral bioavailability [85]. Novartis and BMS also reduced the peptidic character of the P_3-P_2 moiety to yield compounds **51** and **52** respectively [86, 87]. No reports on further development of compounds **50 - 52** could be found.

Merck entered the tripeptidomimetic series by removing the keto amide TSA moiety of the previously described compound **26**. Although this removal resulted in a fifty fold loss of thrombin inhibitory potency, compound **53** was still a potent thrombin inhibitor, but showed a poor oral activity [88, 89]. Two lines can be distinguished in Mercks optimization from compound **53**. In the first line lipophilic bis-aryl moieties like D-diphenylalanine, 3-3-dicyclohexyl propionate, 3-3 diphenyl propionate and 2-hydroxy fluorenyl were introduced at the D-Phe position [23, 88 - 90]. The bioavailablities observed in this series showed large variations from one species to an other. 2-Hydroxyfluorenyl derivative **54** gave an oral bioavailability of 6% in rats, 39% in monkeys and 74% in dogs [89]. The already mentioned 3-3-diphenyl propionate analog of **54** was reported to have only 10% bioavailability in rats but 58% in dogs [90]. Another major obstacle was encountered at this phase of the optimization program. The lipophilicity (or the hydrofobicity) of the compounds increased in order to get oral absorption but this had detrimental effects on the antithrombotic potential [23]. It is a delicate trade-off between hydrofilicity and *in vivo* efficacy on one hand and hydrofobicity and oral absorption on

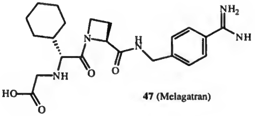
Table 7. **Tripeptidomimetics Strategy A: Maintain high basicity**



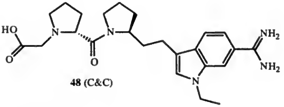
2



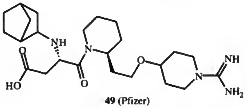
46 (Inogatran)



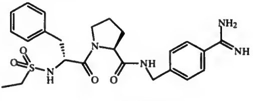
47 (Melagatran)



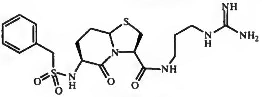
48 (C&C)



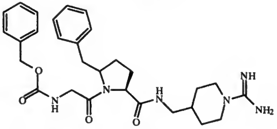
49 (Pfizer)



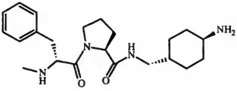
50 (Eli Lilly)



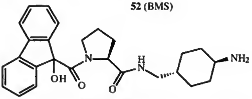
51 (Novartis)



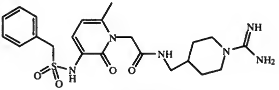
52 (BMS)



53 (L-371912)



54 (L-372460)



55 (L-373890)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
2	0.182			
46	0.015	32-51		dose dependency in po activity
47	0.002		>50	
48	0.016*	33		
49	0.0041			Trypsin Ki = 1.2 μM
50	(770.10^6 M^{-1})**	12		
51	0.145			
52	none given			
53	0.005	5-10	5-10	
54	0.0015	6	74	monkey: F = 39%
55	0.0005			monkey: F is low

* IC_{50} chromogenic assay. **K_{ass}

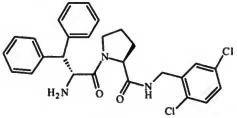
the other hand. In the second optimization line the complete P_2-P_3 moiety was exchanged for a pyridinone moiety [91]. This resulted in a moderately active thrombin inhibitor. Introduction of the benzylsulfonamide and amidinopiperidine moieties gave the potent and selective but not orally active thrombin inhibitor **55**.

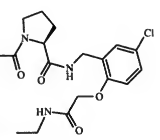
Optimization Strategy B: Reduce High Basicity

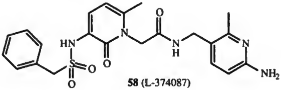
Merck used the well-optimized P_2-P_3 moieties found in strategy A as new starting point to discover P_1 moieties of reduced or no basicity (Table 8). With D-diphenylalanine-proline as P_2-P_3 moiety the result was an orally bioavailable compound: **56** [92]. However, despite a good *in vitro* thrombin inhibitory potency compound **56** performed poorly in a thrombosis model. This poor *in vivo* potency is due to the high lipophilicity of **56** [93]. Optimization aiming at reduction of the lipophilicity resulted in compound **57** [93]. Also the pyridinone acetamide moiety of compound **55** was used to explore new P_1 moieties. This resulted in the selective and efficacious thrombin inhibitor **58** which displayed a good oral bioavailability but modest half-lives [94, 95]. Further studies to optimize the pharmacokinetic profile of **58** yielded pyrazidone **59** which was selected for further clinical development [96]. No reports have been found on the outcome of the clinical evaluation of **58**. The 1-aminopyridine moiety was also used by Eli Lilly in combination with a type of P_3-P_2 moiety that was successful in strategy A resulting in compound **60** [97]. In this case no data concerning oral bioavailability was reported.

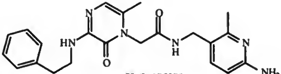
The non-peptide P_3-P_2 scaffold used by Merck was first used in elastase inhibitor design by researchers at Zeneca [98]. Corvas and Organon

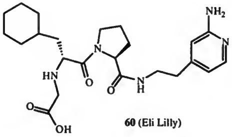
Table 8. Tripeptidomimetics Strategy B: Reduce High Basicity

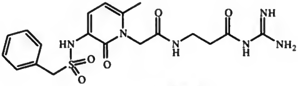

56 (Merck)


57 (Merck)


58 (L-374087)


59 (L-375378)


60 (Eli Lilly)


61 (Organon)

Compound	Ki IIa (μM)	rat F (%)	dog F (%)	Remarks
56	0.003		45	poor *in vivo* potency
57	0.00074	10	40	monkey F = 63%
58	0.0005	< 5	44	monkey F = 19%
59	0.0008	42	91	monkey F = 60%
60	12×10^6 M^{-1} **			
61	0.029*			

*calculated Ki, ** K_{ass}

also used these moieties in the same time span resulting in compounds **11** and **61**, the pyrimidinone and the pyridinone acetamide scaffold respectively [38, 99]. In addition, analog **61** also has a less basic-acylguanidine-moiety (pKa of 7.6) as an isosteric replacement for guanidine. No oral bioavailability was reported with regard to compound **61.**

In conclusion, both strategies have successfully addressed the lack of thrombin inhibitory potency in the lead compound **2** and delivered orally bioavailable compounds. In strategy A the most advanced and promising compound Melagatran (**47**) was the result of introducing a carboxylate at the N-terminus. In strategy B the L-375378 is the most promising compound. This compound is the result of an aminopyridine moiety of low basicity at the P_1 position combined with a non-peptidic P_2-P_3 moiety.

SERIES 3. ARGATROBAN-TYPE COMPOUNDS

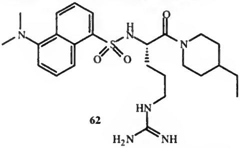
The argatroban-type series can broadly be characterized as a group of thrombin inhibitors in which the carboxylate of the central amino acid is functionalized to a tertiary amide and the α-position is substituted with an aryl sulfonamide moiety.

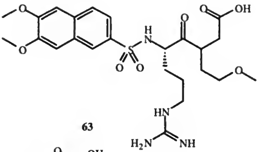
Optimization Strategy A: Maintain High Basicity

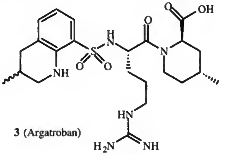
A typical example of this optimization strategy is the discovery of argatroban itself. Starting from TAME (Fig.(**2**)) the potent thrombin inhibitor **62** was obtained (Table 9) [19]. However, the high toxicity of this compound made it unsuitable for clinical use. The strategy used to reduce the toxicity of compound **62** is well-explained by Haijikata-Okunomiya and Okamoto in a review concerning argatroban. They stated: " Since the arginine was necessary for keeping the thrombin inhibitory activity, the remaining two pods were open to chemical modifications to reduce the toxicity. .... Since the modification of the amide portion of C-terminal side did not affect the thrombin inhibitory activity, the effect of COOH introduction into the amide portion was studied." [19] This strategy resulted in compound **63** which was a potent and selective thrombin inhibitor with a reduced toxicity. Additional optimization (fine tuning) resulted in a safe compound without oral activity: argatroban which has demonstrated its clinical potential as i.v. antithrombotic agent [100].

To obtain thrombin inhibitors with good pharmacokinetic properties Stürzebecher *et al.* used 3-TAPAP (**64**) as the starting point for optimization

Table 9 **The design route to Argatroban**


62

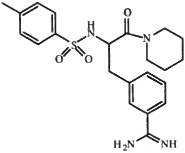

63

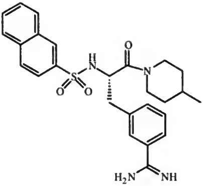

3 (Argatroban)

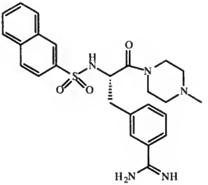
Compound	IIa IC_{50} (µM)/Ki (µM)	Mice intraperitoneally LD (mg/kg)
62	0.1 / 0.037	80
63	0.3 / NR*	900
3	0.1 / 0.019	550

*NR: not reported

Table 10 Benzamidine Analogues


64 (3- TAPAP)


65 (NAPAMP)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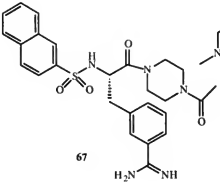
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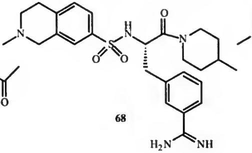
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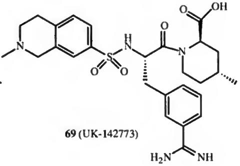
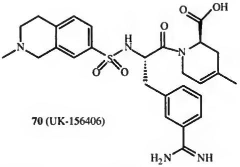
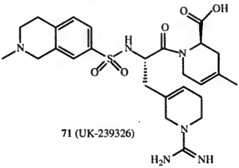

69 (UK-142773)


70 (UK-156406)


71 (UK-239326)

Compound	Ki IIa (μM)	Ki Trypsin (μM)	Dog F (%)	Remarks
3 (Argatroban)	0.010			TT; IC_{50} = 0.062 μM
64	0.34	1.2		
65	0.0025	0.042		TT; IC_{50} = 0.24 μM
66	0.016	0.47		TT; IC_{50} = 0.22 μM
67	0.012	0.044		TT; IC_{50} = 0.062 μM
68				hemodynamic toxicity
69	0.0031	0.054		well-tolerated
70	0.00046	0.026	45	Phase I
71	0.00006	0.050		orally active

(Table 10). Since X-ray crystal analysis of the 3-TAPAP-thrombin complex indicated space for modification both at the toluene and piperidine moiety these moieties were varied [101, 102]. However, despite the improved thrombin inhibitory activity of compounds **65** and **66**, they both displayed poor anticoagulation activity (prolongation of clotting times of human plasma) which is probably due to binding to plasma proteins [103]. Furthermore, these compounds did not show interesting pharmacokinetic properties [103]. Acetylpiperazine **67** displayed a thrombin inhibitory potency and an anticoagulant activity that is similar to argatroban. However it is doubtful whether the properties of compound **67** will be sufficient for application as an orally active antithrombotic since the plasma levels in rats after oral administration at an high dose (50 mg/kg) compared with the levels after i.v. administration (1 mg/kg) indicate a modest oral bioavailability.

Furthermore, the high potency in trypsin inhibition could lead to side effects [103].

Argatroban, compound **62** and benzamidine based compounds such as 3-TAPAP (**64**) inspired researchers at Pfizer to prepare compounds like **68** that showed a good thrombin inhibitory potency but some also showed toxic effects [104]. Introduction of a carboxyl group at the same position of the piperidine ring as in argatroban (the free-site) yielded compound **69**, which turned out to be a potent thrombin inhibitor and was well-tolerated in mice. The pharmacokinetic properties and potency were improved further by introduction of unsaturation at the piperidine ring. UK-156406 (**70**) showed an improved duration of action due to a decreased hepatic clearance probably as a result of reduced lipophilicity. In dogs, UK-156406 displayed a plasma half-life of 0.8 h and an oral bioavailability of 45%. This compound is currently in Phase I studies. Since only a moderate selectivity against trypsin is observed, the structure was further optimized (fine tuning) which resulted in UK-239326 (**71**). The latter compound showed an improved thrombin inhibitory potency and selectivity against trypsin and an *in vivo* profile similar to UK-156406.

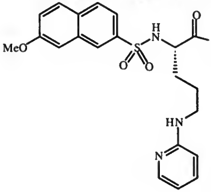
Optimization Strategy B: Reduce High Basicity

Researchers at Bristol-Meyers Squibb replaced the highly basic guanidine moiety of argatroban by heteroaryl moieties of attenuated basicity to improve oral bioavailability. They simplified the arylsulfonyl and piperidine moiety for evaluation of this concept [105] (Table 11). Indeed the permeability across Caco-2 cell monolayers (an *in vitro* model of intestinal absorption) was improved for these heteroaryl based compounds compared to their guanidine counterparts, but the most potent compound (**72**) was still 10-fold less active than argatroban. To our knowledge, the next step in the optimization strategy, improve/restore thrombin inhibitory activity, was not reported.

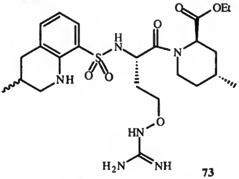
Introduction of an oxoguanidine moiety and esterfication of the carboxylate of argatroban yielded compound **73** which gave a 10-fold reduction of antithrombotic potency but with a significantly improved Caco-2 cell permeability. However, despite the improved permeability no oral activity was observed in mice (dose 50 mg/kg).

In contrast to **72** and **73,** hydroxyguanidine analogue **74** showed good thrombin inhibitory

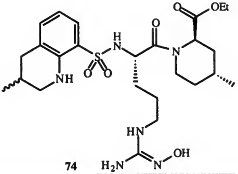
Table 11. Analogues of Argatroban with P_1 Moiety of Reduced Basicity



72



73



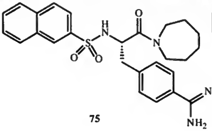
74

Compound	pKa	IC50 IIa (μM)	Caco-2; Papp (nm/s)
3 (Argatroban)	13	0.04	3
72	7	0.47	30
73	7	1.30	67*
74	9	0.05	19*

*Value is estimated from a bar diagram of the Caco-2 Papp values activity but a poor Caco-cell permeability [106]. Furthermore, the latter compound did not show any meaningful *in vivo* activity after i.v. or p.o. administration to mice. It should be noted that esterfication of the carboxylate of argatroban alone or substitution of the guanidine of argatroban by a less basic guanidine moiety as such gave compounds of poor Caco-2 permeability. It appears that the basic guanidine and carboxylate moieties both are determinants of the poor permeability of argatroban [106].

Researchers at LG Chemical exploited the amino acid difference at position 190 (S pocket) in thrombin and trypsin and prepared thrombin inhibitors with an improved selectivity towards trypsin (Table 12). Replacement of the benzamidine moiety (pKa ~12) of compound **75** by benzamidrazone (pKa =8.9) resulted in a potent and selective thrombin inhibitor **76** [107]. In this case two steps, introduction of an isoster of low basicity to improve the profile and improve/restore the thrombin inhibitory potency, were achieved by one modification. However, the limited water solubility of compound **76** hampered further development. Fine-tuning of the tertiary amide resulted in the potent and selective thrombin inhibitor LB-30057 (**77**) that not only displayed a good water solubility but also a good oral bioavailablity in dogs [108]. Furthermore, LB-30057 is an effective antithrombotic agent in a canine model of venous thrombosis [109]. Recently, several analogues of LB-30057 were described in which the naphthyl group was replaced by various hydrophobic

Table 12. Compounds Prepared at LG Chem



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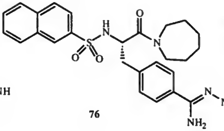
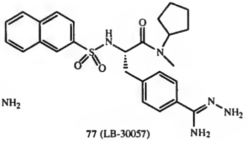
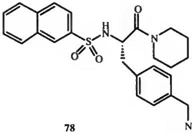


76



77 (LB-30057)



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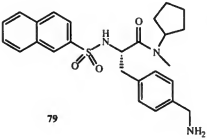

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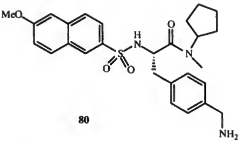
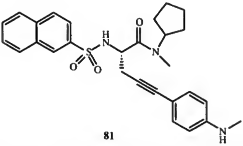
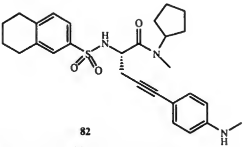


80



81



82

Compound	Ki IIa (μM)	Ki Trypsin (μM)	Rat F (%)	Dog F (%)	Remarks
75	0.052	8.7			
76	0.0015	3.7			aq. sol. << 1 mg/ml *
77	0.00038	3.3		58	aq. sol. >>10 mg/ml *
78	3.1	24			
79	0.018	13,6			Caco-2:Papp = 110 nm/s
80	0.0066	14,2	77		Caco-2:Papp = 107 nm/s
81	0.019	37			
82	0.005	> 30	orally available		

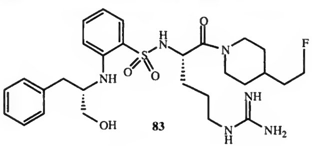
* aq. sol.: aqueous solubility

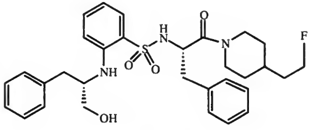
moieties. These compounds showed an enhanced thrombin inhibitory potency without sacrificing the selectivity over trypsin and the pharmacokinetic profile [110].

Besides the benzamidrazone group also the benzylamine group (pKa=9.4) was explored as replacement for benzamidine [111]. This replacement was investigated before by Stürzebecher *et al* but their best benzylamine based inhibitor **78** showed only a modest activity [112]. Researchers at LG Chemical used a solid phase synthesis to prepare a large number of compounds and **79**, again a N,N-cyclopentylmethyl derivative, turned out to be a potent and selective thrombin inhibitor that displayed a good Caco-2 cell permeability [111, 113]. Exploration of aryl sulfon amide moieties, which were comparable or superior in thrombin affinity to the naphthyl sulfon amide moiety in the study of benzamidrazones, yielded compounds such as **80** that showed a further increased in thrombin inhibitory potency [111]. Moreover, several of these compounds displayed oral bioavailablity in rats.

The structure activity relationship information on LB-30057 was used in the investigation of new N-(arylsulfonyl)propargylglycinamide derivatives as

Table 13. Compounds Prepared at Novartis


83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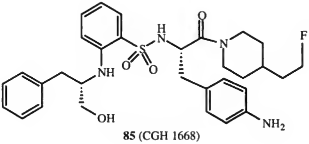
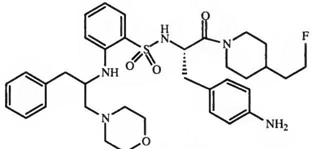

85 (CGH 1668)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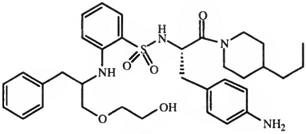
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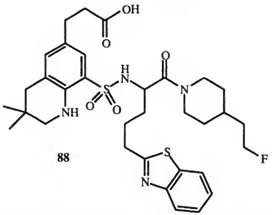
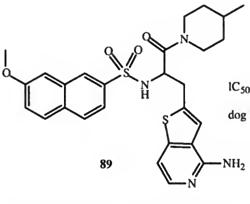

88

Compound	Ki IIa (μM)	Rat F (%)
83	0.016	no oral bioavailability
84	0.10	orally bioavailable
85	0.013 - 0.039	12
86	0.022	61
87	0.147	55
88		50

thrombin inhibitors [114]. In the first design cycle the naphthylsulfonyl and N,N-cyclopentylmethylamidyl were kept constant and a variety of neutral or mildly basic moieties were explored as acetylenic substituents. N-methyl p-aminophenyl as substituent exhibited good thrombin inhibitory activity (compound **81**) and in the following design cycle the arylsulfonyl moiety was optimized. This resulted in the potent and selective thrombin inhibitor **82** which displayed oral bioavailability in rats (no $T_{1/2}$ or F described in literature).

Also researchers of Novartis discovered in this series of argatroban-type compounds p-aminophenyl based thrombin inhibitors such as the lead compound CGH1668 (**85**) [115] (Table 13). Their studies started with arginine based compound **83**. This compound is a potent thrombin inhibitor but displayed no oral bioavailability. To improve the oral bioavalability the highly basic guanidine moiety was replaced by a new P_1 pharmacophore of attenuated basicity. In the design of this new P_1 residue the lipophilicity of the top part of the S pocket was exploited. This resulted in the moderately potent thrombin inhibitor **84**, which showed oral bioavailability. Since this compound is quite lipophilic (ClogD = 3.8) the optimization was directed towards more polar compounds. Introduction of an amino group resulted in the discovery of CGH1668 (**85**), a orally bioavailable, selective and potent thrombin inhibitor. Also some optimization studies (fine-tuning) of CGH1668 were described [116, 117]. In these optimizations the influence of the structure on the thrombin inhibitory activity (Ki and APTT) and on the absorption (blood samples from the portal vein and cartoid artery) were determined. This resulted in the discovery of orally bioavailable and selective thrombin inhibitors such as compounds **86** and **87**. Also neutral P_1 moieties were explored by Novartis [118]. The orally bioavailable compound **88** resulted from this exploration [7].



$IC_{50} = 0.53\ \mu M$

dog F = 36%

89

Fig. (6). Aminothieno[3,2c]pyridine prepared by Organon.

In our group we explored aminobicycloaryl moieties at the P_1 position in the argatroban-type series. This exploration resulted in thrombin inhibitors like compound **89** (Fig.(**6**))[119]. This compound showed a good oral bioavailability in dogs [120].

In conclusion, in the argatroban type series both strategies were successful in several cases. Maintaining the highly basic moiety (strategy A) yielded several interesting compounds for clinical applications such as argatroban (**3**), UK-156406 (**70**) and UK-239326 (**71**). It should be noted that in all of these compounds introduction of a carboxylate was the crucial step in the optimization process. Also reduction of the basicity of the P_1 moiety was successful in this series and compound like LB-30057 (**77**), compounds **83** - **88** described by Novartis and compound **89** from Organon show progress towards the ideal antithrombotic.

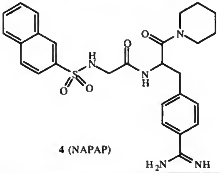
SERIES 4. NAPAP-TYPE COMPOUNDS

The NAPAP-type series class can be characterized as inhibitors in which the carboxylate of the central amino acid is functionalized with a tertiary amine and the α-position is substituted with a N-aryl sulfonyl amino acid. NAPAP is the lead compound in this series. This compound is a potent thrombin inhibitor but with some well known disadvantages like: short plasma half life, lack of oral bioavailability, and side-effects *in vivo* [21].

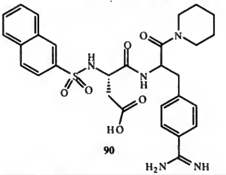
Optimization Strategy A: Maintain High Basicity

Researchers at Behringwerke improved the characteristics of NAPAP by introduction of an acid function (Table 14) [121, 122]. They scanned NAPAP for a free site to attach an acid and found that substitution of NAPAP's glycine by aspartate (compound **90**) led to an acceptable loss of thrombin inhibitory activity and to reduced side effects *in vivo* (influence on blood pressure and histamine release). Optimization of the aryl sulfon amide moiety yielded CRC-220 (**91**), a potent and selective thrombin inhibitor. Despite a reasonable stability in plasma and gut homogenates and a reasonable Caco-2 cell permeability (passive and active transport) CRC-220 exhibited a low oral bioavailabilty due to a large first-pass elimination by carrier-mediated transport in hepatocytes [123, 124].

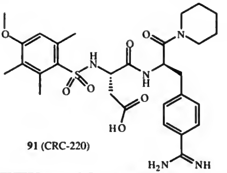
Table 14 Optimization of NAPAP (4) by Introduction of a Carboxylate



4 (NAPAP)



90



91 (CRC-220)

Compound	Ki IIa (μM)	Ki Trypsin (μM)	Caco-2; Papp (nm/s)	Rat F (%)
4	0.011	499		
90	0.020	505		
91	0.0024	312	12.6	4

Optimization Strategy B: Reduce High Basicity

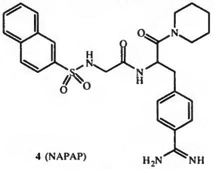
In our group we were also intrigued by NAPAP's antithrombin potency and wanted to improve its profile by replacement of the benzamidine moiety with an isoster of reduced basicity. Inspired by the crystal structures of the NAPAP-thrombin complex we decided to investigate 1-aminoisoquinoline as benzamidine isoster in NAPAP [125] (Table 15). The resulting compound **92** showed a considerable reduction in thrombin inhibitory activity but a significant improvement in Caco-2 cell permeability was obtained. The next step in our optimization strategy was to regain the thrombin inhibitory potency by optimisation of the N-naphthylsulfonyl glycine moiety. This resulted in the potent thrombin inhibitor **93** (Org 37476). In addition, the selectivity over trypsin and the Caco-cell permeability were improved considerably.

In conclusion, strategy A seems to yield potent compounds like CRC-220 (**91**) but this compound lacks sufficient oral bioavailability. In strategy B the potent thrombin inhibitor Org 37476 (**93**) was obtained.

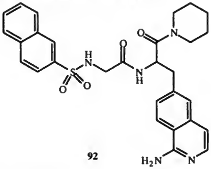
THROMBIN INHIBITORS FROM SCREENING

The majority of the LMW thrombin inhibitors can be classified as derived from fibrinogen, and fall within the four series described before. In general, these inhibitors still have the amino acid framework. One strategy is to use the information obtained from crystal structures of thrombin and use in silico-screening to generate novel non-peptide inhibitors. The discovery of XU 817 (**94**) is a typical example of such a strategy [126] (Fig. (**7**)). In silico-screening started with 5-(amidino)indole as arginine mimic and modeling gave suggestions for the optimal way to interact with the P and D pockets. This resulted in the potent and selective thrombin inhibitor XU 817, which showed efficacy in the rat vena cava thrombosis model (a model for venous thrombosis)

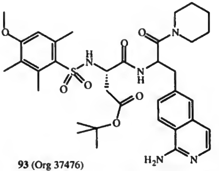
Table 15. Optimization of NAPAP (4): Reduction of Basicity



4 (NAPAP)

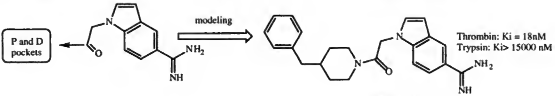


92



93 (Org 37476)

Compound	IC_{50} IIa (μM)	IC_{50} Trypsin (μM)	Caco-2; Papp (nm/s)
4	0.69	0.17	4
92	55	255	19
93	0.08	378	50



94 (XU 817)

Fig. (7). XU 817.

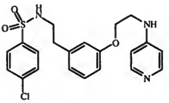
after i.v. administration. Although the structure of XU 817 is quite different from the compounds in the fibrinogen derived series some features such as the amidino function and a kind of amino acid backbone can still be recognized.

Screening of compound libraries, however, presents the opportunity for a radical departure from amino acid based leads and has delivered some novel leads in the search for the ideal thrombin inhibitor. In general, these leads from screening showed a low to moderate thrombin inhibitory activity and the first efforts in optimizations were devoted to improve their potency without sacrificing their unique structural features. In the following section optimizing three of these leads from screening is described.

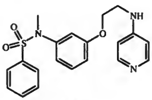
Diarylsulfonamides and Esters

Researchers at Boehringer Mannheim discovered the achiral 4-aminopyridine derivative **95** from screening, which inhibited thrombin competitively and selectivity [127] (Table 16). The first goal of their optimization was to improve the potency of **95** without impairing its selectivity [24, 127, 128]. Maintaining the low-basic aminopyridine moiety (pKa = 9.2) and varying of the remaining part of **95** yielded a 40 fold improvement in thrombin inhibitory potency (compound **96**). From these optimization studies and later confirmed by crystallographic studies it became evident that the pyridine moiety interacted with the S-pocket, the two phenyl moieties interacted with the P-pocket and D-pocket and no moiety interacted via hydrogen bonding with Gly^{216} of thrombin. In

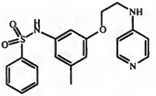
Table 16. Diarylsulfonamides and Esters



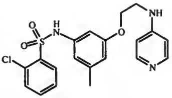
95



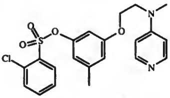
96



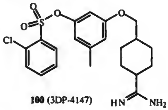
97 (BM 141248)



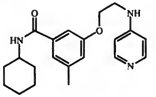
98



99



100 (3DP-4147)



101

Compound	Ki IIa (μM)	Ki Trypsin (μM)	Dogs F (%)
95	2.8	> 500	
96	0.07	> 500	
97	0.023	> 500	60 - 70
98	0.023		
99	0.011	11	
100	0.0046	25	orally active
101	0.008*		

* IC_{50}

conclusion, these novel inhibitors not only have unique structural features but also an unique binding mode [129]. In addition, **97** showed a good pharmacokinetic profile in dogs [24].

Researchers at 3-Dimensional Pharmaceuticals were intrigued by the non-peptide aminopyridine based thrombin inhibitors described above. The aminopyridine **99** disclosed by Boehringer Mannheim was used in a study aiming to improve the thrombin inhibitory activity [130]. Analysis of the crystal structure **99**-thrombin complex made them decide to prepare a series of analogues of compound **99** in which the aminopyridine was replaced by guanidine containing moieties in order to improve potency. Although many of these guanidine based compounds showed a lower potency, they also managed to improve the inhibitory potency in a some cases. One of the resulting compounds, 3D-4147 (**100**), displayed an excellent bioavailability in dogs [131]. Also Glaxo Wellcome investigated this type of inhibitors as illustrated by their disclosure of compound **101** [132].

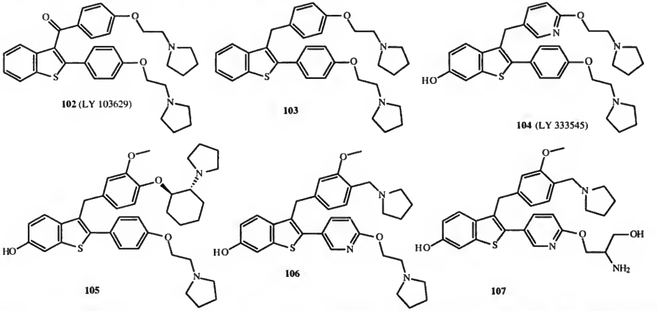
Dibasic Benzo[b]thiophene Derivatives

Screening efforts at Eli Lilly resulted in the identification of benzo[*b*]thiophene LY 103629 (**102**) as a thrombin inhibitor [133] (Table 17). Although this compound showed only weak thrombin inhibition, its novel structure, selectivity over trypsin and oral bioavailability are intriguing. Early SAR studies identified the structural elements responsible for thrombin affinity and led via compound **103** to the design of LY 333545 (**104**). The latter compound exhibited a 50 fold enhancement in thrombin inhibition over LY 103629, a good selectivity over trypsin and a moderate oral bioavailability in rats. Furthermore, crystallographic studies of the thrombin- LY 103629 complex confirmed the uniqueness of this series of thrombin inhibitors since its binding is quite novel compared to the amino acid based inhibitors. Recently, inhibitors such as **105-107** were disclosed that displayed an *in vitro* sub-nanomolair potency and antithrombotic efficacy in a rat AV-shunt model [134, 135]. To our knowledge no pharmacokinetic data of these compounds were released.

Lactones

Screening at Glaxo Wellcome identified GR133686 (**108**) as a potent thrombin inhibitor [136] (Table 18). This compound inhibited thrombin by acylation of Ser^{195} of thrombin's catalytic triad via the strained lactone ring.

Table 17. Benzo[*b*]thiophene Derivatives



102 (LY 103629)

103

104 (LY 333545)

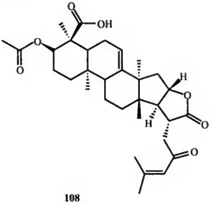
105

106

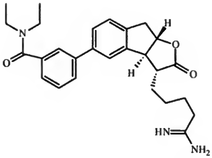
107

Compound	Kass IIa (10^6L/mole)	Ki IIa (μM)	Rat F (%)
102	0.43	0.37	15
103	3.4		52
104	24	0.0099	9
105	309		
106	1182	0.0005	
107	668	0.0055	

Table 18 Lactones



108



109

Compound	IC_{50} IIa (μM)	APTT (μM)*	Remark
108	0.04		
109	< 1	4	not stable in plasma

*APTT (2x control)

Simplification of the structure, introduction of the highly basic amidine group and optimization of the lipophilic interactions with thrombin gave lactones like compound **109** [137]. Although some of these compounds showed a good *in vitro* thrombin inhibitory activity their instability in plasma hampered further development.

In conclusion, sreeening yielded some leads with quite different chemical features than the leads derived from fibrinogen. Compounds like BM 141248 (**97**), 3DP-4147 (**100**) and LY 333545 (**104**) clearly show the potential of this approach.

CONCLUSIONS

It is difficult to judge the full potential of all compounds described in this review. Using the limited *in vivo* data disclosed we have selected the most promising ones based on their oral bioavailability and depicted them in Fig. (**8**). Interestingly, more than one strategy has proven to be successful in achieving its final goal. Compounds resulting from strategy A are generally more polar than ones resulting from strategy B. This can be a potential advantage in *in vivo* antithrombotic efficacy. In strategy A the most rewarding theme seems to be the introduction of a carboxylate at the proper position. The recurrent theme of an orally bioavailable thrombin inhibitor combining a basic moiety with a carboxylate indicates to a specific transport mechanism, possibly a charge compensation phenomenon or a carrier mediated form. The only report on the involvement of a carrier in these zwitterionic thrombin inhibitors is for CRC-220 (**91**) but not for absorption from the intestine but for hepatic clearance [123, 124]. Nevertheless, it has been shown that if amidine or guanidine groups are neutralized by a carboxylate the compounds may become less toxic. This was illustrated by the optimization program that resulted in argatroban (**3**), UK-239326 (**71**) and CRC-220 (**91**).

In the strategy B significant progress has been made in recent years. The hurdle of finding moieties of low basicity that can substitute for the amidine or guanidine has been taken. However, in most cases this has resulted in an increased lipophilicity which may jeopardize the *in vivo* efficacy of such thrombin inhibitors. An other theme in these inhibitors and some inhibitors from screening is the occurrence of aminoaryl moieties as P_1 moiety (e.g. 2-aminopyridine in L-375378 (**59**), N-methyl aniline in compound **82**, aniline in LB-30057 (**77**), 4-aminothieno[3,2c]pyridine in compound **89**, 1-aminoisquinoline in Org 37476 (**93**) and 4-aminopyridine in BM 141248 (**97**)).

As far as we know there are currently no compounds beyond phase I clinical trials with p.o. administration protocols. Although it might be too early to say that the ideal antithrombotic has finally been discovered, it is clear that the compounds depicted in Fig. (**8**) have overcome the major hurdle for further development in this area: oral bioavailability. These compounds clearly illustrate the power of lead optimization which has produced several valid solutions for the bioavailablity-antithrombotic dilemma.

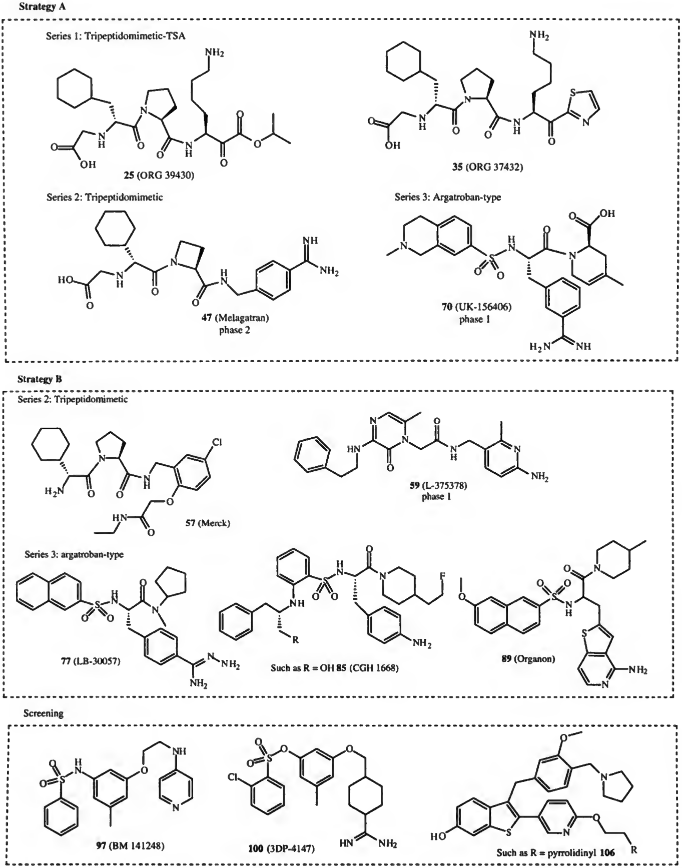

Strategy A
Series 1: Tripeptidomimetic-TSA
25 (ORG 39430)
35 (ORG 37432)
Series 2: Tripeptidomimetic
47 (Melagatran)
phase 2
Series 3: Argatroban-type
70 (UK-156406)
phase 1
Strategy B
Series 2: Tripeptidomimetic
57 (Merck)
59 (L-375378)
phase 1
Series 3: argatroban-type
77 (LB-30057)
Such as R = OH 85 (CGH 1668)
89 (Organon)
Screening
97 (BM 141248)
100 (3DP-4147)
Such as R = pyrrolidinyl 106

Fig. (8). Selection of promising compounds or type of compounds based upon published thrombin inhibitory activity and oral bioavailability data.

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Approaches to the Prevention of Coronary Vascular Dysfunction Caused by Myocardial Ischaemia and Reperfusion

Owen L Woodman*

Department of Pharmacology, University of Melbourne, Parkville 3052 Victoria, Australia

Abstract: The endothelium is an important regulator of coronary vascular tone due to its ability to release potent vasoactive substances such as the vasodilators nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF), prostacyclin (PGI_2) and the potent vasoconstrictor endothelin. Endothelial dysfunction has been associated with a number of pathological states such as atherosclerosis, hypertension, diabetes and congestive heart failure. A disturbance of endothelial function may also contribute to the adverse effects that ischaemia and reperfusion exerts on the coronary vasculature. After ischaemia and reperfusion there is usually a selective impairment of endothelium-dependent relaxation in isolated coronary arteries. However, in the intact coronary circulation, there is a general loss of vasodilator reserve as responses to both endothelium-dependent and endothelium-independent agonists are attenuated. The release of vasoconstrictor(s) and plugging of capillaries with leukocytes may contribute to that impairment of the capacity of the coronary circulation to dilate together with the reduction in basal blood flow (no-reflow phenomenon). Ischaemic preconditioning is able to prevent ischaemic damage to the myocardium but the vasculature is less well protected as reperfusion is enhanced but the vasodilator reserve continues to be limited. Pharmacological preservation of vascular function has proved more successful with inhibitors of leukocyte adhesion, calcium channel blockers, endothelin receptor antagonists and inhibitors of oxygen radical generation all offering protection. Further refinement of protocols to preserve endothelial and vascular function after ischaemia will aid reperfusion, enhance vasodilator reserve and maximise recovery of myocardial function.

INTRODUCTION

Ischaemia can cause significant tissue damage and cell death when the interruption to blood flow is prolonged. It is now appreciated that in the heart this damage is not limited to the cardiac muscle but may also involve the coronary vasculature. As the main aim in the treatment of ischaemia is to restore blood flow or to 'reperfuse' the ischaemic vascular bed impaired vascular function may prevent that goal being attained. Thus, the common observation upon reperfusion after a prolonged period of ischaemia is that there is a sustained reduction in blood flow, in comparison to pre-ischaemic levels, which is referred to as the 'no-reflow' phenomenon. If thrombolysis and percutaneous transluminal coronary angioplasty (PTCA) are to provide the maximum benefit in the treatment of acute myocardial ischaemia [1] it is important that we improve our understanding of the effects of ischaemia on the vasculature. We may then be able to identify targets for pharmacological treatment that will maximise reperfusion.

The endothelium is now recognised to be an important regulator of vascular tone rather than a simple barrier between the blood and smooth muscle. As a site of synthesis and release of potent vasodilators such as nitric oxide (NO), endothelium-derived hyperpolarising factor (EDHF) and prostacyclin (PGI_2) together with the vasoconstrictor endothelin the endothelium is an important tissue to consider when the consequences of myocardial ischaemia and reperfusion are investigated. In particular the interaction between the endothelium and leukocytes upon reperfusion offers a key target for intervention to improve coronary vascular function after ischaemia.

THE EFFECT OF ISCHAEMIA AND REPERFUSION ON CORONARY VASCULAR FUNCTION

When assessing the effect of myocardial ischaemia and reperfusion on coronary vascular function most studies assess reactivity in isolated vessels *in vitro* [2, 3]. There are both advantages

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*Address correspondence to this author at the Department of Pharmacology, University of Melbourne, Parkville 3052 Victoria, Australia; Fax: 613 9347 1452; Email: o.woodman@pharmacology.unimelb.edu.au

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and limitations to such studies. By using isolated tissue endothelial and smooth muscle function may be assessed separately. In addition potentially complicating (extravascular) modulators of arterial tone, for example blood flow, transmural pressure and neuronal or circulating vasoactive factors, are eliminated. However, the vessels used in such studies are usually large and would offer little resistance to blood flow *in vivo*. They may therefore fail to adequately represent changes that occur in the microvasculature which is critical in controlling myocardial perfusion. To more accurately determine the likely consequences of ischaemia and reperfusion to coronary vascular reactivity observations from isolated tissue experiments need to be considered in conjunction with data from *in vivo* studies.

VASCULAR REACTIVITY *IN VITRO*

Most studies using isolated arterial rings indicate that ischaemia and reperfusion causes a selective impairment of endothelium-dependent relaxation [2, 3]. Not all studies have reported that ischaemia impairs endothelium-dependent relaxation of conduit arteries [4, 5] and Quillen *et al* [4] also noted that although ischaemia did not affect relaxation of larger arteries there was a significant impairment of endothelium-dependent relaxation in the microvasculature. In contrast, endothelium-independent relaxation is consistently preserved after ischaemia/reperfusion indicating that smooth muscle function is not affected.

VASCULAR REACTIVITY *IN VIVO*

The vascular consequences of ischaemia and reperfusion may be more precisely defined from studies performed in the whole animal. We and others [2, 3] have observed that after a period of myocardial ischaemia and reperfusion there is a significantly lower level of blood flow to the previously ischaemic myocardium in comparison to the pre-occlusion level of flow. Furthermore there

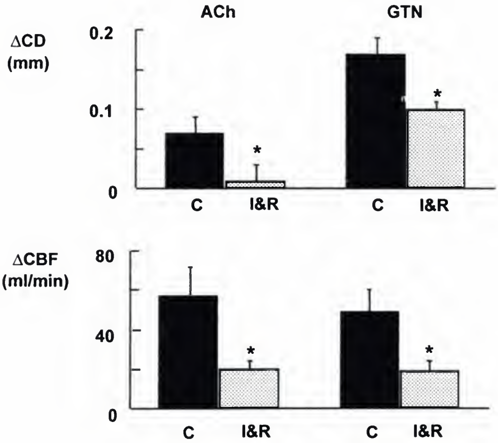

Fig. (1). Mean increases in left circumflex coronary artery diameter (CD, n=5, top panel) and coronary blood flow (CBF, n=10, bottom panel) in anaesthetised dogs in response to the intra-coronary injection of the endothelium-dependent vasodilator acetylcholine (ACh, 0.02 mg/kg) or to the endothelium-independent vasodilator glyceryl trinitrate (GTN, 0.2 mg/kg). The control responses (C, solid bar) are significantly greater than the responses following 60 min of occlusion of the coronary artery and 30 min of reperfusion (I&R, shaded bars). *$P<0.05$ compared to control, Student's paired t-test. From Sobey *et al.* [6].

is a significant reduction in the diameter of the circumflex coronary artery, with no change in arterial pressure, indicating active constriction of the ischaemic epicardial artery [2, 6]. In addition, we [6-8] and others [9] have reported that the reduction in myocardial perfusion is accompanied by a reduction in vasodilator reserve. This is indicated by a reduction in responses to both endothelium-dependent and endothelium-independent vasodilators that occurs in both the conductance and resistance vessels Fig. (**1**). Examination of the microvasculature in the ischaemia/reperfusion zone by electron microscopy revealed stasis of red blood cells in capillaries and plugging of capillaries by leokocytes [6]. Such capillary plugging would also contribute to a reduction in vasodilator reserve.

The observations in the intact circulation, taken together with those in the isolated arteries, suggest that while ischaemia and reperfusion cause endothelial damage, that damage is only one factor contributing to the changes in vascular reactivity. The reduction in diameter of the epicardial coronary arteries that occurs in the absence of any change in arterial pressure [6] indicates that a vasoconstrictor may be released, which then opposes responses to exogenous vasodilators. For example, the circulating levels of endothelin are reported to be significantly increased after ischaemia and reperfusion in experimental studies and in humans [10] and this could lead to active vasoconstriction.

It should be noted that impaired responses to acetylcholine usually have been taken to indicate

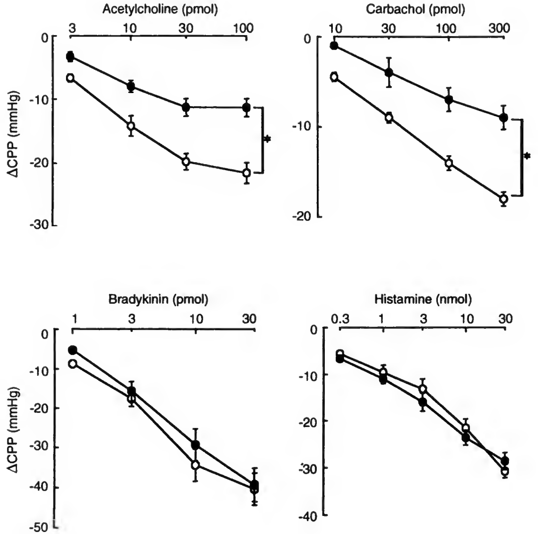

Fig. (2). The effect of 30 min low flow-ischaemia and 15 min reperfusion on the responses to endothelium-dependent vasodilators in rat coronary resistance arteries preconstricted with U46619. The vasodilator responses to acetylcholine (top-left; control n=9, ischaemia n=6) and carbachol (top-right; control n=4, ischaemia n=4) but not to bradykinin (bottom-left; control n=5, ischaemia n=6) or histamine (bottom-right; control n=5, ischaemia n=6) were significantly impaired by 30 min low flow ischaemia-reperfusion (filled circles) as compared to responses in control hearts (open circles). * $P < 0.05$ compared to time control group, ANOVA. From Pannangpetch & Woodman [12].

general endothelial dysfunction and often that is not confirmed by inhibition of responses to other dilators acting in an endothelium-dependent manner. We have examined the effect of ischaemia on responses to a variety of endothelium-dependent vasodilators in rat isolated hearts and rat hindquarters perfused with physiological solution [11, 12]. In those studies, where the function of resistance vessels was examined, ischaemia impaired responses to muscarinic receptor agonists such as acetylcholine and carbachol but did not affect responses to other endothelium-dependent dilators such as bradykinin and histamine Fig. (**2**). Similarly Kim *et al.* [13] observed that although ischaemia did affect responses to bradykinin, the level of impairment was significantly less than that seen with acetylcholine. Thus, muscarinic dilator mechanisms may be particularly sensitive to the effects of ischaemia and perhaps the use of different agonists can indicate the degree of endothelial dysfunction.

An alternative explanation to the variable sensitivity of different agonists is that there may be a variable contribution of different endothelium-derived mediators to those agonists together with different susceptibility of those mediators to the effect of ischaemia. Thus it is now appreciated that in addition to nitric oxide (NO) a non-NO endothelium-derived hyperpolarising factor (EDHF) also mediates endothelium-dependent vasodilatation [14].

CONTRIBUTION OF NO AND EDHF TO ENDOTHELIUM-DEPENDENT RELAXATION AFTER ISCHAEMIA AND REPERFUSION.

The endothelium regulates vascular tone by the release of a number of factors including endothelium-derived relaxing factor (EDRF), characterised as nitric oxide (NO) [15], EDHF [14, 16], which remains to be identified, and prostaglandins such as PGI_2 [17]. EDHF opens potassium channels to hyperpolarise cells leading to closure of voltage-operated calcium channels to cause relaxation [14, 18]. The type of potassium channels involved remains unclear but the calcium-sensitive potassium channels on the ATP-sensitive potassium (K_{ATP}) channels are most commonly proposed to be involved [14, 18]. The contribution of EDHF to relaxation is not always apparent and in some studies it was reported to contribute to relaxation only when the NO system is blocked [19, 20]. This suggests that EDHF may operate as a complementary system that assumes greater importance when the NO system is impaired which, for example, can occur in artery diseases [18]. Hence the contribution of EDHF to vasodilatation may be increased where there is endothelial dysfunction such as in the presence of hypercholesterolaemia, congestive heart failure [21] and perhaps after myocardial ischaemia and reperfusion.

Myocardial ischaemia/reperfusion has generally been reported to cause selective attenuation of the endothelium-dependent relaxation of isolated coronary arteries [6, 22-24] without impairing the function of the smooth muscle cells. However, Quillen *et al.*, [4] and Winn & Ku [25] both reported that ACh-induced relaxation of dog coronary arteries was unaffected by ischaemia and reperfusion. This then raises the question as to whether EDHF might contribute to endothelium-dependent relaxation after ischaemia and reperfusion to help to maintain responses to endothelium-dependent dilators. We investigated the effect of 60 min of coronary artery occlusion and 120 min reperfusion *in vivo* on subsequent endothelium-dependent relaxation of dog coronary arteries *in vitro* [5]. Ischaemia/reperfusion did not affect the responses to the endothelium-dependent vasodilator acetylcholine. In addition the responses to acetylcholine in non-ischaemic and ischaemic coronary arteries were equally impaired by inhibition of NO synthase or soluble guanylate cyclase. This indicated that ischaemia did not alter the contribution of NO or cyclicGMP to the endothelium-dependent relaxation. However, whereas responses to acetylcholine in non-ischaemic arteries were unaffected by depolarisation with high K^+, in ischaemic arteries the same treatment caused a significant reduction in the endothelium-dependent relaxation. Tetraethyl ammonium (TEA) but not glibenclamide, an inhibitor of K_{ATP} channels, significantly reduced endothelium-dependent relaxation in ischaemic arteries indicating that the opening of large conductance potassium (BK_{Ca}) channels was involved in the relaxation Fig (**3**). Thus, in addition to considering the effect of ischaemia and reperfusion on the magnitude of vasodilator responses, determination of effects on the mechanism of relaxation may offer insight to the changes in vascular function that are observed.

There are no other reports of the effect of ischaemia on the mechanisms of endothelium-dependent dilatation. However, the results of this study are similar to the observations of Cohen and his colleagues [26, 27] who investigated the effect of hypercholesterolaemia on the mechanism of

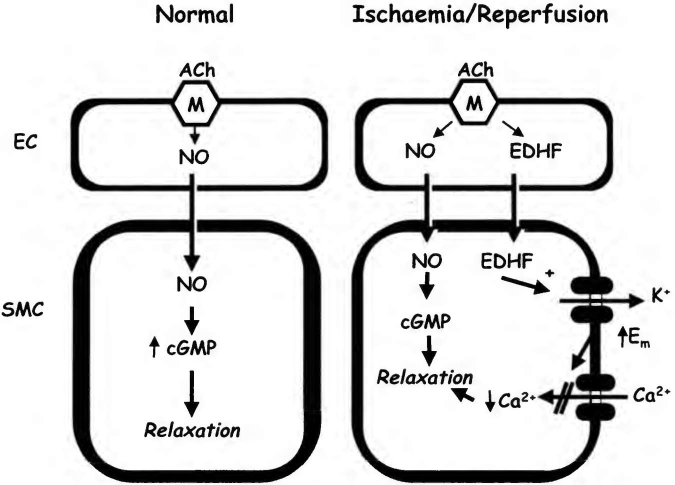

Fig. (3). In the normal dog epicardial coronary artery (left) acetylcholine causes relaxation by releasing NO which acts on soluble guanylate cyclase to increase cGMP. There is no evidence to suggest that hyperpolarisation is involved as the relaxation is unaffected by high K^+ (67 mM) or the BK_{Ca} channel blocker TEA (1 mM). By contrast after ischaemia and reperfusion endothelium dependent relaxation may be impaired by both inhibition of NO synthase (L-NOARG) and by high K+ and TEA. Furthermore the actions of TEA and L-NOARG were additive [5] suggesting that both NO and a non-NO EDHF contributed to the relaxation.

endothelium-dependent relaxation of rabbit carotid arteries. They reported that hypercholesterolaemia did not affect the relaxant responses to acetylcholine nor was the sensitivity of that relaxation to inhibition of NOS different between normal and hypercholesterolaemic arteries. However elevated K^+ impaired acetylcholine-induced relaxation in hypercholesterolaemic, but not normal arteries [26]. Further it was demonstrated that in hypercholesterolaemic arteries charybdotoxin also inhibited the endothelium-dependent relaxation indicating that it was the BK_{Ca} channel that was involved in the relaxation [27]. Furthermore, Malmsjo *et al.* [28] have recently reported that the contribution of EDHF to endothelium-dependent dilatation is also enhanced in congestive heart failure, another disease state associated with impairment of endothelium-dependent vasodilation [29]. Thus it appears that pathological states such as ischaemia/reperfusion, as well as hypercholesterolaemia and congestive heart failure, may alter the mechanism of endothelium-dependent relaxation even in the absence of any change in the magnitude of the relaxation. Thus ischaemia and reperfusion triggers a number of events that may then influence vascular function (Table 1) in terms of the basal perfusion, vasodilator reserve and the mechanism of endothelium-dependent vasodilatation.

PRECONDITIONING AGAINST VASCULAR DYSFUNCTION

The myocardium may be protected from the adverse effects of ischaemia and reperfusion by

Table 1. Key Events in the Adverse Effects of Ischaemia and Reperfusion on Vascular Function

Event	Consequence
Impaired NO synthesis/release	Selective impairment of endothelium dependent relaxation *in vitro*
Enhanced role for EDHF	Reserve mechanism for endothelium-dependent vasodilatation
Release of endothelin and perhaps other vasoconstrictors	Constriction of epicardial and resistance coronary arteries
Adhesion of leukocytes to the coronary endothelium	Capillary plugging and restriction of reperfusion
Oxygen radical generation	Vascular (and myocardial) tissue injury, impaired endothelium-dependent vasodilatation

prior exposure to single or repeated brief periods of ischaemia and reperfusion [30, 31]. This phenomenon, known as ischaemic preconditioning, reduces myocardial infarct size, decreases the number and severity of arrhythmias upon reperfusion and improves recovery of ventricular function [30]. Despite the well defined benefits of ischaemic preconditioning on the myocardium the effect on blood vessels has not received the same attention and it remains less certain that preconditioning preserves coronary vascular function from the adverse effects of ischaemia and reperfusion.

The majority of studies examining the effect of preconditioning on endothelial and vascular function have been performed using isolated tissues. Thus, in isolated hearts perfused with physiological saline, preconditioning prevents the endothelial dysfunction that normally occurs after ischaemia/reperfusion [32-34]. Similarly, preconditioning preserved endothelium-dependent relaxation in pig coronary arterioles [35] and dog large coronary arteries [36] isolated from previously ischaemic myocardium. Such improvements in endothelial function caused by preconditioning are consistent with the observation that brief periods of coronary artery occlusion lead to a delayed enhancement of endothelium-dependent vasodilatation associated with an upregulation of NO synthesis [37]. However, as discussed above, there are limitations to the use of *in vitro* techniques when investigating the effects of ischaemia on the vasculature as there is a selective impairment of endothelium-dependent relaxation in isolated tissues but a general reduction in vasodilator reserve *in vivo*.

When examining coronary vascular function *in vivo* there are several reports that preconditioning does increase the level of blood flow upon reperfusion [8, 38, 39] but the ability of preconditioning to preserve vasodilator reserve is less certain. At present, there are only three reports examining the effect of ischaemic preconditioning on coronary vasodilator reactivity *in vivo*. Bauer *et al.*, [40] found that in the dog coronary vasculature, four cycles of brief ischaemia and reperfusion had no effect on either endothelium-dependent or endothelium-independent dilatation. Our observations in anaesthetized dogs were similar [8]. We found that ischaemic preconditioning was able to improve the level of resting blood flow upon reperfusion and to reduce infarct size. However vasodilator responses to acetylcholine and the endothelium-independent vasodilator glyceryl trinitrate were impaired to a similar extent after ischaemia and reperfusion with or without prior exposure to ischaemic preconditioning Fig (**4**). Thus even when the ischaemic preconditioning was clearly effective in preventing damage to the myocardium there was no beneficial effect upon vasodilator reserve after reperfusion. In contrast, DeFily and Chilian [41] demonstrated that prevention of the impairment of acetylcholine and serotonin-induced dilatation of the coronary vasculature was achieved with only one cycle of brief ischaemia and reperfusion in the dog. However, in that study, ischaemia and reperfusion failed to impair endothelium-independent dilatation as is most commonly observed *in vivo* [2, 3]. Perhaps ischaemic preconditioning preserves the ability of the endothelium to mediate vascular relaxation but does not prevent the activation of other events that reduce vasodilator reserve such as leukocyte adhesion and vasoconstrictor release. Surprisingly, a study in the rat isolated, blood-perfused heart demonstrated that global myocardium ischaemia impaired the decrease in coronary vascular resistance induced by sodium nitroprusside, an endothelium-independent vasodilator and that attenuation was prevented by ischaemic preconditioning [42]. That study indicated that the impairment of vascular smooth

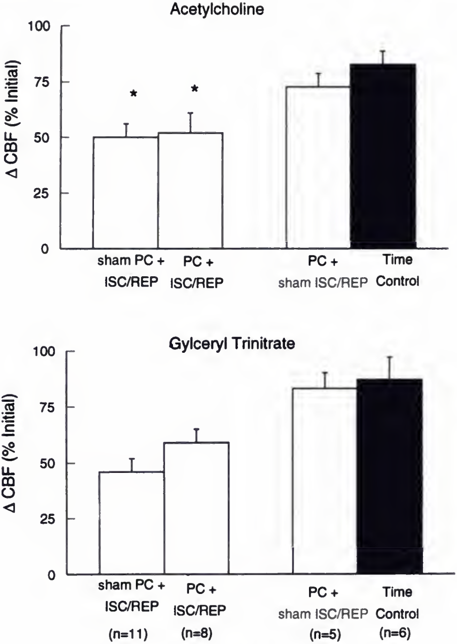

Fig. (4). The effect of left circumflex coronary artery (LCx) occlusion on coronary vascular responses to acetylcholine (ACh: 1-30 ng/kg, top) and glyceryl trinitrate (GTN: 30-300 ng/kg, bottom). Vasodilator responses were expressed as a percentage of initial changes in coronary blood flow (CBF) and one average value was obtained from all doses of each vasodilator. Time control (*n*=6) and preconditioning alone (PC: 2 cycles of 5 min occlusion and 5 min reperfusion and 1 cycle of 5 min occlusion and 10 min reperfusion; *n*=5) had little effect on vasodilator responses to either ACh or GTN. By contrast 60 min LCx occlusion and 60 min LCx reperfusion (ISC/REP; *n*=11) significantly impaired vasodilator responses to both ACh and GTN compared to time controls. However this impairment was not prevented by preconditioning (*n*=8) even though the preconditioning significantly improved coronary blood flow after reperfusion and reduced infarct size.. Values are mean ± s.e.mean. $^*P < 0.05$, Student's unpaired *t*-test. From Loke & Woodman [8].

muscle function after severe ischaemia and reperfusion can be prevented by ischaemic preconditioning. However, those authors also reported that after ischaemia and reperfusion the response to serotonin was converted from dilatation to constriction, an action which reflects impairment of endothelium-dependent dilatation. The vasoconstriction was also observed in preconditioned hearts indicating that preconditioning appears to have, at best, a limited ability to preserve vasodilator function. The absence of improvement in dilator reactivity in the study of Loke and Woodman (1998) is particularly surprising given the improvement in resting blood flow. The increase in basal perfusion may result from the reduction in infarct size and, thus, a larger proportion of the myocardium is perfused. In addition, ischaemic preconditioning has been reported to reduce leukocyte adhesion and migration upon reperfusion [43] which may reduce capillary plugging and thus aid reflow. The absence of a corresponding increase in vasodilator reserve suggests that the cause of the reduced capacity to dilate is different to that leading to the lower resting blood flow. It is possible that the reduction in vasodilator reserve is the result of active constriction of coronary resistance vessels such as that reported in the epicardial arteries [6] and thus a functional antagonism of dilator responses. Thus at present the evidence for a beneficial effect of ischaemic preconditioning on vascular function *in vivo* is limited at best and requires further investigation. Pharmacological treatment may prove to be a more effective means to prevent ischaemic vascular dysfunction.

PHARMACOLOGICAL PREVENTION OF ISCHAEMIC VASCULAR DYSFUNCTION

The importance of the interaction of neutrophils with the endothelium in the development of ischaemia-induced vascular dysfunction is supported by studies investigating the means of preventing such injury. For example, removing neutrophils from the blood prior to ischaemia reduces coronary endothelial dysfunction [44], as does inhibition of neutrophil adhesion [45]. Nitric oxide donors also prevent ischaemic vascular and myocardial dysfunction [46-48], and this effect is accompanied by a reduced accumulation of neutrophils in the reperfused myocardium. Nitric oxide is known to inhibit the adhesion of neutrophils to the endothelium, and that action may contribute to the preservation of endothelial and vascular function after ischaemia and reperfusion. Interestingly, recent studies have demonstrated that heparin treatment is able to prevent the ischaemia-induced impairment of coronary dilator responses to acetylcholine in anaesthetised dogs [49, 50]. The beneficial effects of heparin do not appear to be reliant on anticoagulant activity as the improvement of function was also apparent after treatment with N-acetyl heparin which has no anticoagulant activity [50]. Rather the beneficial actions of heparin may be associated with activation of the NO-cGMP pathway as there were elevated nitrate/nitrite levels in the great cardiac vein and elevated levels of cGMP in the coronary artery [49, 50].

Inhibition of the effects of oxygen-derived free radicals has also proved to be an effective means of preserving coronary vasodilator capacity after ischaemia and reperfusion. For example, treatment with the xanthine oxidase inhibitor allopurinol before ischaemia and reperfusion prevents impairment of endothelium-dependent and -independent dilatation *in vivo* [7] and preserves endothelium-dependent relaxation of isolated coronary arteries [24]. Oxygen radical scavengers such as superoxide dismutase also prevent ischaemic vascular dysfunction [51]. 17β-Oestradiol, which is known to have antioxidant activity [52], has also been reported to prevent ischaemia-induced coronary vascular and myocardial dysfunction in anaesthetised dogs. The beneficial effects on vascular and myocardial function were accompanied by evidence of enhanced NO synthesis and inhibition of oxygen radical generation and lipid peroxidation. Given the epidemiological evidence that the incidence of coronary artery disease is lower in pre-menopausal women than in age-matched men and that oestrogen replacement therapy lowers the risk of cardiovascular mortality in post-menopausal women [53] further studies into the mechanism of action of oestrogen in the prevention of ischaemic vascular dysfunction are warranted, particularly after long term administration.

The benefit of calcium antagonists in the treatment of ischaemic heart disease is well established [54] and in experimental studies calcium antagonists improve postischaemic myocardial function [55]. In anaesthetised dogs, we demonstrated that the long-acting dihydropyridine calcium antagonist amlodipine improves post-ischaemic vascular function [7, 24]. Amlodipine prevented the reduction in basal coronary blood flow normally observed after ischaemia reperfusion [7]. In addition, responses to both endothelium-dependent and endothelium-independent dilators improved, demonstrating that amlodipine attenuated the ischaemia-induced reduction in vasodilator reserve. Part of the protective effect of amlodipine may involve a preservation of endothelium-dependent vasodilatation, which was observed when vascular function in the previously ischaemic coronary arteries was assessed *in vitro*. However, that effect would not explain the improved responses to the endothelium-independent nitrovasodilator observed in the intact circulation. A possible mechanism for the protective actions of calcium antagonists may be inhibition of neutrophil function. Calcium antagonists have been shown to inhibit neutrophil activation and the subsequent release of superoxide anions [56], an action that may not rely on the inhibition of calcium influx. Furthermore, nisoldipine has been reported to inhibit leukocyte adhesion and sequestration in the coronary microcirculation induced by the activating agent N-formylmethionylleucylphenylalanine (fMLP) [57]. Thus, it is possible that calcium antagonists may inhibit ischaemia-induced activation and accumulation of neutrophils upon reperfusion, an action that would help to maintain vasodilator reserve.

Endothelin may also contribute to some of the adverse consequences of ischaemia and reperfusion. For example, after ischaemia and reperfusion plasma levels of endothelin are elevated and there is an increased sensitivity to the coronary vasoconstrictor actions of endothelin that may be due to an upregulation of endothelin receptors [10]. The adverse role of endothelin in ischaemia and reperfusion is supported by observations that endothelin receptor antagonists prevent ischaemic injury to both the myocardium and coronary vasculature. For example, endothelin receptor antagonists reduce infarct size in pigs [58] and improve post-ischaemic recovery of contractility in rat isolated hearts [59]. In both studies the protection of the myocardium was accompanied by an improvement in coronary blood flow during reperfusion. Once again, inhibition of the ischaemia-induced interaction between neutrophils and the endothelium may be a key event in the beneficial actions of the endothelin receptor antagonists. Endothelin stimulates neutrophil adhesion to cultured endothelial cells and accumulation in perfused hearts [60]. Gonon *et al.* [61] reported that an endothelin receptor antagonist reduced ischaemia-induced impairment of contractility only when neutrophils were included in the perfusate. In addition, when the perfusate contained neutrophils, the presence of the endothelin antagonist increased the outflow of the neutrophils from the heart indicating reduced adhesion to the coronary endothelium. Furthermore Szabo *et al.* [62] found that, in a heterotopic rat heart transplantation model, endothelin receptor antagonists improved coronary blood flow during early reperfusion of the transplanted heart and significantly enhanced endothelium-dependent dilatation compared to untreated hearts. Thus endothelin receptor antagonists may prevent ischaemia-induced vascular and myocardial function. As endothelin acts on two receptor subtypes ET_A, which mediates vasoconstriction and ET_B, which mediates NO release as well as vasoconstriction, the selectivity of the antagonist may be significant. Non-selective or ET_A selective antagonists are usually reported to prevent ischaemic injury [58, 59, 61] whereas an ET_B receptor antagonist was reported to be ineffective [59]. However selective ET_A and ET_B receptor antagonists were reported to preserve endothelial function [62]. Further studies are required to clarify the mechanism of the protective actions of the endothelin receptor antagonists.

Thus a diverse group of pharmacological agents are able to preserve vascular function after ischaemia and reperfusion. Despite that apparent diversity they do all appear to be linked by an ability to reduce the interaction between the endothelium and neutrophils upon reperfusion supporting that process as a key event leading to impaired vascular function after ischaemia.

CONCLUSIONS

In addition to the myocardium, the coronary vasculature is an important site of ischaemia-induced injury, resulting in impaired reperfusion and reduced vasodilator reserve that may impair postischaemic cardiac function. Although results of experiments conducted in isolated arteries have suggested that endothelial damage is a key event in ischaemia-induced vascular dysfunction, it is apparent from studies in the intact circulation that it is only one of the factors that may lead to impaired reperfusion of the myocardium. The adhesion of neutrophils to the endothelium in the coronary microcirculation may cause a physical obstruction to blood flow. In addition, neutrophils are the source of a vasoconstrictor that may increase coronary vascular resistance and oppose vasodilatation [63]. Endothelial dysfunction may of course contribute to the neutrophil adhesion as both NO and PGI_2 inhibit neutrophil activation and adhesion [64] and endothelin released from the endothelium may stimulate neutrophil adhesion [60].

Thus, it is proposed that the interaction of the endothelium with neutrophils provides a key target for the prevention of ischaemia-induced vascular dysfunction and for the improvement of reperfusion. A variety of methods have been employed to prevent ischaemic vascular injury such as ischaemic preconditioning, antioxidants/oxygen radical scavengers, calcium channel antagonists, endothelin receptor antagonists and agents that prevent neutrophil adhesion. These treatments may all be linked by the ability to improve endothelial function, nitric oxide synthesis and/or to inhibit neutrophil activation and adhesion to endothelial cells. Pharmacological treatment that limits the adhesion of neutrophils to the endothelium that is stimulated by ischaemia and reperfusion may improve myocardial reflow, preserve vasodilator reserve and maximise revovery of myocardial contractility after acute coronary ischaemia.

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